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ABSTRACT

Studies during the last granting period were focussed on the activating ability of tumor Ag identified from HER-2 and FBP for T cells from breast cancer patients. Ongoing studies focusses on improvement of Ag presentation and enhanced activation of CTL functions concludes that: (1) IL-12 is required for early and rapid IFN- induction in response to tumor Ag. This was established using both randomly selected breast cancer patients, and patients vaccinated with HER-2 tumor Ag, E75 using GM-CSF as adjuvant; (2) Tumor Ag such as E75 induce the angiostatic/anti-angiogenic chemokine IP-10 from responder lymphocytes (3). Enhanced Ag presentation and immunogenicity can be achieved by inducing Ser/Thr phosphorylation of HER-2.

Abbreviations used: Folate Binding Protein, FBP, Folate receptor α = FR-α, Dendritic cells, DC, T cell receptor, TCR, Interferon-induced protein-10, IP-10, Interleukin-12, IL-12, Protein/Receptor Tyrosine Kinase, PTK, RTK), Phosphorylation, P-tion, okadaic acid, OA, sodium orthovanadate, VAN, neu differentiation factor, NDF, epidermal growth factor, EGF;

INTRODUCTION

Development of biological therapies for cancer in recent years has generated new hopes that improved cancer cure rates can be achieved beyond what is currently obtained with combination of chemotherapy and radiation therapy. Biological therapies use tumor Ag and cytokines (or their genes) with the objectives: **(1)** to induce tumor specific CTL and augment Ag presentation to anti-tumor effectors, **(2)** to achieve optimal proliferation and expansion of specific anti-tumor effector T cells (1), and **(3)** to ensure full activation of anti-tumor immunity.

Since CTL epitopes from tumor antigens are short peptides generated from self-proteins, to address the first objective, ongoing studies needed to accomplish general major tasks: **(1)** to characterize the tumor Ag either by mapping with synthetic peptides or by sequencing of the naturally processed peptides presented by the tumor **(1, 2), (2)** to defnine approaches for presentation of these epitopes to tumor reactive CTL; **(3)** to identify the ability of tumor Ag presented in different forms (either as peptides, or genes encoding the peptides) to induce functional activation of responder $CD8^+$ cells in terms of cytolysis and cytokine production. **(4)** to enhance tumor Ag presentation.

The objective of our studies during this grant period were five-fold: (1) to expand and apply the knowledge regarding stimulation with peptide tumor Ag plus IL-12 learned from model work with healthy donors to breast cancer patients; (2) to establish whether recognition of tumor Ag by $CD8^+$ cells induce the anti-angiogenic/angiostatic chemokine IP-10; (3) to complete the studies on recognition of HER-2^+ tumors by cellular immune effectors by addressing the involvement of natural immunity and immunosurveillance; (4) to complete the studies on antigenicity (recognition) and immunogenicity (activatory ability) of Folate Binding Protein (FBP)/Folate Receptor -α(FR-α) and its relationship with the increase in certain TCRVβ families; (5) to characterize tumor Ag presentation by the tumor (ovarian and breast) by identifying approaches to enhance this presentation.

The reasons for this investigation were:

(1) to complete the studies on tumor Ag recognition by cellular immune effectors. We investigated in parallel with CTL recognition the recognition by Natural Killer Cells which define the first line of defense to tumors and immune surveillance.

(2) to complete the studies on tumor Ag recognition, by investigating the immunogenicity of tumor Ag: we followed two directions of research (a) to investigate the outcome of *in vivo* (in patient) stimulation with tumor Ag (E75-HER-2) and define the effects of IL-12 in potentiating this response; and (b) an alternative approach is to perform *in vitro* stimulation with tumor Ag of tumor infiltrating lymphocytes (TIL) to induce/enhance specific CTL effectors. Then the specific CTL can be used for adoptive immunotherapy. This was performed in the Folate Binding Protein (FBP) system. In parallel a limited analysis of T cell receptor (TCR) Vβ families was performed, to establish whether focus on certain families can be beneficial. Active cancer vaccination (a) and adoptive immunotherapy with in vitro stimulated by tumor Ag effectors (b) are two complementary approaches to cancer immunotherapy. The first (a) may be more suitable for patients with disease remission, where the organism can develop an immune response, while the second may be more suitable for patients with advanced disease where for different reasons (e.g. tumor mediated suppression) the organism cannot mount an immune response.

(3) CTL have been described as mediating cytolysis and cytokine production. Since IP-10 (interferon induced protein - 10) attracts effectors at the tumor site and inhibits blood vessels formation, if tumor Ag can mediate chemokine induction this will be an added anti-tumor effect of potential significance for breast cancer treatment.

(4) to complete the characterization of tumor Ag presentation by breast and ovarian tumors. This is because HER-2 as well as other tumor Ag is phosphorylated by interaction with growth factors. We characterized the role of phosphorylation (P-tion) in Ag presentation and recognition. Thus our studies address the question of the relationship between Tyrosine (Tyr) and Serine (Ser) P-tion and Ag presentation and functional activation by breast and ovarian tumors.

The results of these studies and their significance is presented in the Body of the report.

Body of the Report.

MATERIALS AND METHODS

SKBR3.A2 cells were cultured in RPMI+10% FCS in E75 plates, 12h before addition of agonists the medium was changed to 1% FCS as described. Targets were incubated with agonists in AIM-V medium, for 3h (which does not contain EGF/EGF-like factors, (per GIBCO - technical service information), ^{51}Cr labeled then washed three times and tested in CTL assay.

Cytokines. The following cytokines were used in this study: GM-CSF (Immunex corp., Seattle), specific activity 12.5 x 10^7 CFU/250mg, IL-4 (Biosource International), specific activity 2 x 10^6 U/mg, IL-2 (Cetus Emeryville, CA), specific activity 4 x 10^6 BRMP U/mg, IL-15 (Genzyme, Cambridge, MA), specific activity 2 x 10^6 U/mg.

Synthetic peptides. Peptides were synthesized in the Synthetic Antigen Laboratory of U.T. M. D. Anderson Cancer Center using solid phase techniques on an Applied Biosystems 430 peptide synthesizer (Applied Biosystem, Foster City, CA). Identity and purity of final material were established by amino acid analysis and analytical reverse phase HPLC (Rainin). All peptides utilized in this study were between 92-95% pure. Two FBP peptides were selected for synthesis based on the presence of leucine, isoleucine or valine in the dominant anchors position. As there previously reportd recognition by TAL the peptides position and sequence are as follows: E39 (FBP, 191-199) EIWTHSYKV; E41 (FBP, 245-253) LLSLALMLL. Both peptides are low to moderate binders to HLA-A2[1].

Cells. For induction of dendritic cells in the presence of cytokines GM-CSF and IL-4, HLA.2+ PBMC were obtained from healthy donors from the Blood Bank of M.D. Anderson Cancer Center. For generation of DC by the CD14 method, PBMC were distributed in 24 well plates at 4 x 10^6 cells/well in RPMI 1640 medium. After 2 h of incubation, the nonadherent cells were removed. Complete RPMI medium containing 1000 U/mL GM-CSF and 500 IU/mL IL-4 was added to each well and the adherent cells were cultured for 5-7 days, while they developed the DC characteristic morphology.

T cell stimulation by peptide pulsed DC. DC were washed three times with serum free medium, plated at 1.2 x 10^5 cell/well in 24-well culture plates and pulsed with FBP peptide. E39, at 100 g/ml in serum free medium for 4 hours before addition of responders as described [2,3]. These DC were designated as DC-E39. Paralled control DC cultures were establiished and maintained in the exact same manner except for the omission of FBP peptide (designated DC-NP). The responder TAL in complete RPMI medium were added to DC at 3 x 10^6 cells/well (stimulator : responder ratio of 1:25). 16 hours later IL-2 was added to each well at a final concentration of 30 IU/ml and the cultures were left undisturbed for the following 5 days when CTL activity was determinied.

Flow cytometry for TCR Vβ expression. TAL were stained with fluorescein and phycoerythrin-conjugated mAb specific for the TCR Vβ families. The following mAbs were purchased from Pharmingen (San Diego, CA) and Endogen (Wolburn, MA). Vβ3.1, Vβ5a, Vβ6.7, Vβ8a, Vβ9, Vβ12, Vβ13, Vβ17, Vβ23. The normal mouse IgG1 and IgG2a of Ig isotype were used as isotype controls. Two-color flow cytometry CD8:TCR Vβ was performed using a FACScan (Becton-Dickinson) as described [4]. Since there are more than 20 Vβ families, the average percent expression of each TCR Vβ family should be in the range of 4-5%. We considered a significant increase in the percent Vβ for each family when the difference between percent Vβ of DC-E39 stimulated and DC-NP stimulated was higher than 5-10%.

Cytotoxicity assays. Recognition of peptides used as immunogens was performed by standard chromium release CTL assay as described [5]. T2 or tumor targets were labeled with 200 μCi of sodium chromate (Amersham, Arlington Heights, IL) for 1.5 hrs at 37° C, washed twice and plated at 3000 cells/well in 100 μl in 96 well V-bottom plates (Costar, Cambridge, MA). Effectors were added at designated effector:target (E:T) ratios in 100 μl/well. After 5 h of incubation, 100 μl of culture supernatant was collected, and ^{51}Cr release was measured on a gamma counter (Gamma 5500B, Beckman, Fullerton, CA). All determinations were done in quadruplicate. The results are expressed as percent specific lysis as determined by the equation : (experimental mean cpm-spontaneous mean cpm) / (total mean cpm - spontaneous mean cpm) x 100. For peptide-pulsed cytotoxicity assays, the T2 cells were labeled as above, washed, and then incubated either with PBS (T2-NP) or with peptides (DC-E39) for 1.5 hr at 37° C before standard CTL assays were performed.

Cytokine and Chemokine ELISA. IFN- was determined using an ELISA kit (Pharmingen). IP-10 ELISA. The ability of cells to secrete IP-10 in response to FBP peptides was determined by cultureing PBMCs and collecting supernatants at corresponding times. The levels of IP-10 secreted were determined using a modified sandwich ELISA (R&D Systems, Menneapolis, MN). A flat bottom 96-well microtiter plate was coated with 100 μL/well of monoclonal anti-human IP-10 (2 μg/mL in PBS pH 7.2) for 24 h at room temperature. The plate was subsequently washed with PBS pH 7.4, 0.05% Tween-20, then blocked with 3% ovalbumin, 5% sucrose, and 0.05 NaN_3 IP-10 standards were made from recombinant human IP-10 in a solution consisting of Tris-buffered saline (TBS) pH 7.3, 0.05% Tween-20, 0.1% BSA using serial dilutions. 100 μL/well of the standards and the cell supernatants were plated in duplicate and left at room temperature for 2 h. After washing the plate 3 times, 100 μL/well of biotinylated monoclonal anti-human IP-10 (100 ng/mL in TBS pH 7.3, 0.1% BSA) was added followed after washing by 100 μL/well of strepavidin-peroxidase conjugate. Chomogen substrate, 100 μL/well, consisted of DMSO and H2SO4. Plates were read at 450 nm in an automated microplate reader (Bio-Tek Instruments, Inc. Richmond, CA). Standards dilutions of IP-10 ranged from 4,000 pg/mL to 15.6 pg/mL. This method consistently detected IP-10 concentrations greater than 31.25 pg/mL in a linear fashion. (6).

RESULTS

(1) The research performed during the last twelve months has made significant progress toward the goals of this study and the overall goal of developing specific immunotherapy for breast cancer.

1. Effects of tumor Ag on recognition by NK cells

Although natural killer (NK) cells have been described as non-MHC-restricted, new evidence suggests that NK activity can be either up- or down-regulated after interaction with the peptide-MHC-class-I complex expressed on target cells. However, the epitope(s) recognized by NK cells have remained ill-defined. We investigated NK cell recognition of synthetic peptides representing a portion of a self-protein encoded by the HER-2/neu (HER-2) proto-oncogene and presented by HLA-A2. HER-2 nonapeptides C85, E89, and E75 were found partially to protect T2 targets from lysis by freshly isolated and interleukin-2(IL-2)-activated NK cells (either HLA-$A2^+$ or $A2^-$). This inhibition was not solely due to changes in the level of HLA-A2 expression or conformation of serological HLA-A2 epitopes. Using single-amino-acid variants at position 1 (P1) of two HER-2 peptides, we observed that protection of targets was dependent on the sequence and the side-chain. These results suggest similarities in the mechanism of target recognition by NK and T cells. This information may be important for understanding the mechanisms of tumor escape from immunosurveillance and could help explain the aggressiveness of HER-2-overexpressing tumor cells. (Anderson L. et.al. Cancer Immunology Immunotherapy, in press attached).

The conclusion of these studies is that overexpression of HER-2 on breast and ovarian tumors inhibits their recognition by Nk cells. *Thus HER-2^+ tumors are more likely to escape immunosurveillance. Thus the emphasis for anti-cancer therapies focussed on HER-2 should be in induction of a specific response (by CTL and antibodies).*

2. Induction of an effector response by HER-2 CTL epitope vaccination in breast cancer patients require IL-12.

Studies in the last year have demonstrated a requirement for IL-12 in induction of IFN-γ in response to tumor Ag. IL-12 appeared to play both a co-stimulatory role, because it did not induce IFN-γ by itself, and essential because it was required to induce detectable levels of IFN-γ where Ag was present. Results reported last year were obtained with healthy donors. In patients IFN-γ^+ E75-specific cells may be absent, or present at low density. Thus it was important to determine whether breast cancer patients respond in a similar or different way. Two groups of patients were used: (1) from which the blood was drawn at random (all with HER-2^+) tumors (**Fig. 1**) and (2) patients vaccinated with tumor Ag: E75+GM-CSF as adjuvant (**Fig. 2 and 3**).

The results in Figure 1 show that IL-12 was required to induce higher levels of IFN-γ in response to E75, while in two patients (No. 128 and 129) IFN-γ was detected only when IL-12 was added. To clarify whether IL-12 was also required in E75-vaccinated patients we tested the effects of stimulation with E75 ± IL-12 in two patients by testing samples before (pre) and after vaccination (post) (**Fig. 2 and 3**). Complete results from two patients (No. 131 and 132) are shown. These results include cytotoxicity, proliferation and IFN-γ secretion. In one patient (**Fig. 2**) the experiments were repeated with an additional post vaccination sample. The results show that induction of IFN-γ in response to E75 required IL-12 in all samples. The results also show that vaccination with E75 by itself is insufficient to induce specific CTL activity. Based on these findings we are currently designing with Dr. James L. Murray and Dr. Andrejz Kudelka a new trial to include IL-12 in addition to E75 for breast cancer vaccination.

Folate Binding Protein Peptide E39 can activate CTL.

Tumor associated lymphocytes (TAL) isolated from malignant ascites cultured in media containing interleukin-2 show antitumor responses. These antitumor responses are mediated by cytotoxic T lymphocytes (CTL) which recognize antigen in the context of MHC molecules using T cell receptors. $CD8^+$ CTL recognize peptide epitopes processed from cellular proteins in the context of MHC class I molecules. These peptides have a restricted length of 8-11 amino acids. The folate binding protein (FBP) is overexpressed in over 90% of ovarian and 20-50% in breast cancers. We recently found that FBP is the source of antigenic peptides recognize by a number of these CTL-TAL. This indicated that FBP peptides are antigenic in vivo for ovarian and breast CTL-TAL. To define FBP immunogenicity, a peptide defining the epitope E39 (FBP, 191-199) was presented by PMBC derived dendritic cells (DC) from healthy donors isolated by the CD14 method to ovarian and breast CTL-TAL. Stimulation of ovarian and breast CTL-TAL by E39

pulsed DC (DC-E39), in the presence of IL-2, rapidly enhanced or induced E39 specific CTL activity. This E39-responder population consisted of cells expressing TCR Vβ9, Vβ13, and Vβ17 families, based on the increase in the percentages of these families in DC-E39 versus DC-NP stimulated TAL. Characterization of immunogenic tumor antigens and of cytokine requirements for induction of functional antitumor effectors may be important for future cancer vaccine developments. (Kim et-al. Anticancer Research, in press, 1999)

These results show that TAL can be stimulated in vitro by tumor Ag to develop specific CTL effectors. *The results also show that: (1) allogeneic DC can be used as APC for this purpose. This finding may be important since it is not always feasible to obtain DC, or functional APC from breast cancer patients with advanced disease. (2) TAL respond better and faster than PBMC to CTL induction by peptide, apparently because they contain higher numbers of activated Ag-specific CTL; these CTL may be also selected for therapy because they are apoptosis resistant compared with in vitro activated PBMC.*

Induction of IP-10 by recognition of tumor Ag.

The fact that chemokines receptors are expressed on T lymphocytes depending on their state of activation and differentiation suggests that chemokines are involved in efficient attraction of Th1 or Th2 cells at the site of inflammation. Second, the fact that chemokines receptor expression, and association with Th1/Th2 phenotypes can be affected by cytokines known to mediate T lymphocytes polarization, suggest that chemokines may be part of effector and amplification mechanisms and their receptors may seem as targets for selective modulation of T cell dependent immunity (7). Differental expression of chemokines receptors, and patterns of chemokines secretion as well may influence T cell responses other than chemotaxis, as demonstrated by the fact that certain CC chemokines (RANTES, MIP-1∝ and MCP-1) promote lymphocyte activation and/or differentiation.

Individually, chemokines are a large family consisting of four subfamilies that display between two and four highly conserved NH2-terminal cysteine-residues. Most chemokines fall into two groups, CXC and CC. The CXC(∝-family) has the first two NH2-terminal Cys separated by a non-conserved amino acid residue. The CC(β-bamily has these Cys in juxtaposition. The CXC chemokines behave as angiogenic or angiostatic factors depending on the presence of a three aminoacid motif (ELR) which precedes the first conserved Cys residue in the aminoacid sequence. Platelet factor, 4 (PF-04), monokine induced by interferon γ (MiG) and inducible protein-10 (IP-10) are angiostatic factors. Importantly, they are induced by interferons which are known inhibitors of wound repair, especially angiogenesis. Of interest IFN-γ inhibits the production of ELR-CXC, chemokines (angiogenic). *Thus, by shifting the balance in the favor of angiostatic CXC such as IP-10 these effects may be important in regulating net-tumor derived vascularization (8).*

Nothing is known about the ability of tumor Ag to induce chemokines. We found that stimulation of isolated $CD8^+$ cells with E75 induced rapidly, within 24h significant amounts of IP-10. Importantly, this induction did not require additions of exogenous IFN-γ. It was enhanced but not induced by exogenous IL-12 when used at 100 pg/ml (**Fig. 4 A,B**). IP-10 was induced in the absence of antigen at high concentrations (1000 pg/ml) of IL-12. At this time IFN-γ was detectable (~100 pg/ml). These results show that induction of IP-10 by IL-12 alone is a rather slow process. In contrast when lymphocytes were stimulated by E75 in the presence

of IL-12, at the same high concentration (1000 pg/ml), we found significantly higher levels of IP-10 than IFN-γ during the first 24h (**Fig. 5 A,B**). At 48h and 72h the levels of IP-10 were stable and paralleled the levels of IFN-γ induced by stimulation with E75 + IL-12. Thus IP-10 is induced early and rapidly in response to stimulation with tumor Ag. Also, IL-12 sustained higher levels of IP-10 over time.

Induction of IP-10 was mediated through CD40-CD40L interactions, since $\propto$CD40L mAg inhibited IP-10 secretion. These results are shown in **Fig. 6 A and B**). This experiment was performed in the absence of exogenous IL-12. The CD40-CD40L interaction is the major pathway for IL-12 induction in the T cell mediated responses system. The results show that $\propto$CD40L antibody (CD40L is expressed on T cells) inhibited IFN-γ induction essentially completely, in the first 24h, but inhibited at much lesser extent IP-10 induction (by 34%) in the same experiment. Thus as suggested and by the results in **Fig. 5**, IP-10 induction in the first 24h may be not entirely dependent on IFN-γ.

Importantly, induction of IP-10 was enhanced when $CD8^+$ cells were stimulated with Ag variants (E75 variants) suggesting stronger Ag agonists can be obtained by sequence modification. (F42 = S5K, F43 = S5A, F45 = S5G; The first letter indicates the amino acid in the wild-type sequence that was replaced, the numeral indicates the position (e.g. 5) in the sequence while the second letter indicate the replacement. The results are shown in **Fig. 7 A,B**). These results show that the analogs F45 and F43 induce higher levels of IP-10 than the wild-type peptide; the superinduction is Ag-sequence specific, and side chain specific. The wild-type Ag, E75 contains Serine in position 5. Serine has an OH (hydroxyl side chain). F42 contains lysine which expresses a positively charged side chain. In contrast F43 and F45 contain Ala and Gly which lack the HO-group and the -CH2-OH (hydroxymethylene) groups from Serine respectively. *Thus IP-10 induction by E75 appear to be mediated by the interaction/or lack of interaction of the OH group of the Ag with TCR.*

Treatment of tumor cells with HER-2 phosphorylation agonists inhibits tumor Ag presentation. Reversal by TPA and Ser/Thr phosphatase inhibitors.

HER-2 is a transmembrane receptor which mediate intracellular signaling. This intracellular signaling is mediated following phosphorylation (P-tion) of HER-2. Thus the active molecule is

modified at tyrosine by tyrosine kinases and at Ser/Thr by Ser/Thr - kinases (protein kinases). If CTL epitopes are generated from the transmembrane HER-2, then the HER-2 is in most instances either Tyr or Ser/Thr - P-ted. Tumors express P-ted HER-2, because of its involvement in signaling. P-tion at Tyr and Ser/Thr modify the HER-2 chain. For example P-ted Ser mimics Aspartic Acid, while P-tion of Thr mimics glutamic acid. This raises the question whether P-tion of HER-2 affects CTL recognition. The outcome of these effects is important because HER-2 P-tion can enhance tumor recognition or can inhibit tumor recognition. This provides a mechanism for enhanced tumor sensitivity or enhanced tumor escape. To address these questions we investigated the effects of HER-2 agonists EGF (epidermal growth factor), NDF (neu differentiation factor) together with the Tyrosine phosphatase (PTP) inhibitor sodium orthovanadate (VAN) on CTL recognition. In the same time we used PKC activator TPA as an agonist for S/T-P-tion. Futhermore, to stabilize S/T-P-ted groups we used okadaic acid (OA) which is a S/T-phosphatase inhibitor. Thus Y-P-tion should be observed after tumor treatment with EFG+NDF+VAN while S/T-P-tion after treatment with EGF+NDF+TPA, or TPA + OA.

The results are shown below.

1. *TPA and PTP inhibitor (VAN) mediate opposite effects on CTL recognition.* EGF activate RTK and Ser/Thr phosphatases (S/T-P-ases). Down-modulation of RTK is accomplished by endogenous activation of PKC (receptor tyrosine kinases), through the feed-back loop EGF → PLCg → DAG → PKC. PKC is negatively regulated by S/T - P-ase which remove Phospho groups from Ser/Thr. We found that the specific S/T - P-ase inhibitor, OA (50 nM) partially reverted the inhibition of tumor recognition induced by EGF+NDF+VAN. SKBR3.A2 were pre-treated with agonists and washed before addition of CTL. Kinetic analysis of the CTL response was performed to determine whether the agonists' effects are reversible over time. **Fig. 8A** show that while in 4 and 8h CTL assays target pretreatment with EGF+NDF+VAN inhibited lysis by more than 80%, after 20h partial reversal of inhibition was observed, suggesting not only that the agonists effects on tumor are transient, but also that the residual tumor bound agonists do not have direct inhibitory effects on the effector CTL.

2. To address whether tumor pre-treatment with EGF+TPA enhanced recognition by E75 specific CTL, SKBR3.A2 cells were pre-treated either with EGF+NDF+VAN (to enhance HER-2, Y-P-tion and stabilize P-Y groups) or EGF+NDF+TPA to activate the feed-back loop by

inducing Ser/Thr P-tion by PKC (and/or MAPK). EGF and NDF were used at 10-20 ng/ml (1.5-3 nM) respectively, concentrations (10^{-9} M) shown to be mitogenic for breast tumors but not apoptotic or inducing differentiation. TPA was used at 50 ng/ml, concentrations verified to be not toxic in our laboratory. VAN and TPA induced opposite effects in the presence of EGF suggesting central roles for PTP, Y-P-ted HER-2 in regulating epitope presentation **(Fig. 8B)**. Tumor pretreatment with EGF+NDF+VAN decreased lysis of SKBR3.A2 by Donor 1 CTL by 50%, compared with control, while EGF+TPA enhanced lysis by more than 100%. The results were confirmed in a separate experiment where targets were pre-treated with EGF + VAN (to stabilize PY-groups) TPA+OA (to stabilize P-S/T groups). These CTL recognized epitopes in the context of HLA-A2 as indicated by the inhibition of lysis by BB7.2 mAb **(Fig. 8C)**.

3. *TPA+OA pre-treatment of HER-2^{hi} breast tumors enhances their stimulatory ability for primary HER-2 CTL epitope induction.* To address whether enhanced recognition of tumors treated with TPA+OA results in immunogenic presentation of HER-2 epitopes, we determined the ability of SKBR3.A2 pre-treated with EGF+VAN, or TPA+OA to induce primary CTL from another HLA-$A2^{+}$ healthy donor (Donor 5). In SKBR3 cells HER-2 is constitutively phosphorylated at Y-1248. To diminish the possible interference of serum EGF or other factors on epitope presentation SKBR3.A2 cells were cultured in low FCS concentrations (1%) for 12h before addition of agonists for 3h at which time they were washed and cultured in AIM-V medium. After extensive washing, responders, (plastic non-adherent PBMC) were added at a S:R ratio of 1:20. 50U/ml of IL-2 was added 48h later, and recognition of E75 and C85 was determined after 3 additional days. The results **(Figure 4A)** show that Donor 5 T cells simulated with SKBR3.A2 pre-treated with TPA + OA recognized both C85 and E75. **The TPA effects appeared to be mediated by PKC, since T cells stimulated with SKBR3 pretreated with TPA+OA, and the specific PKC inhibitor GF109203X, did not recognize these epitopes.** Recognition of E75 and C85 was not observed by T cells stimulated with EGF+VAN, pre-treated tumor cells, suggesting that induction of tyrosine P-tion does not enhance HER-2 immunogenicity.

Site-directed mutagenesis of Her2/neu. HER-2 mediate oncogenic effects. The main P-tion site involved in HER-2 activation is the Y1248. Replacement of the tyrosine at this site with Phenylanine, which is structurally similar but cannot be P-ted (lacks hydroxyl group), leads to an inactive HER-2 (in terms of oncogenic potential). To develop an inactive HER-2 we

conducted site directed mutagenesis of HER-2, in collaboration with the laboratory of Dr. John Weiner (Department of Molecular Pathology). The approach and the results are shown below:

To generate P-mimetics we first mutated the central Y-P-tion site Y1248 to F1248. The results are shown below. Mutagenesis (Y1248 → F) was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and the primers sense (3895-3935) 5'-CCT ACG GCA GAG AAC CCA GAA TTC CTG GGT CTG GAC GTG CC-3' and antisense (395-3895) 5'-GGC ACG TCC AGA CCC AGG AAT TCT GGG TTC TCT GCC CTA GC'3'. A novel EcoRI restriction endonuclease site (underlined) was created at the same position for the purpose of identifying the mutant. The changes were as follows: GAG → GAA (3914), followed by 3915-TAC(Y) → TTC(F). Thus the F1248+ clones can be identified by sensitivity to EcoRI. To optimize the mutagenesis, a 1.2 kb KpnI fragment was moved from the full-length human cDNA clone into pBluescript. The mutagenesis reactions were prepared and cycled as recommended by the supplier. Following digestion with DpnI to degrade the methylated DNA template, and transformation of E. coli, several potential clones were picked at random and screened for the novel EcoRI site, the presence of which will yield a ~650 bp DNA fragment. **(Fig. 10)** *Ongoing studies are characterizing presentation and recognition of tumor Ag from the innactive HER-2. This may have an important impact on HER-2 vaccination for gene therapy, DNA vaccines for patients with additional HER-2 types because it is not oncogenic.*

Legends to the figures.

Figure 1. IL-12 is required for early and rapid IFN-γ induction in response to tumor Ag, E75.

Figure 2. Immunological monitoring of CTL activity, IFN-γ secretion and increase in the total cell number in PBMC from a breast cancer patient treated with E75 based cancer vaccine. Pre-vacc. indicate pre-vaccine, post-vacc indicate post-vaccine. Please note the increase in IFN-γ production after vaccination in response to E75 and the enhancement by IL-12.

Figure 3. Immunological monitoring of CTL activity, proliferation and IFN-γ secretion in PBMC from another breast cancer patient (No. 132) treated with E75 based cancer vaccine. Post-vacc #2 indicate that the responses were tested again from a second sample. In this patient induction of IFN-γ in response to E75 required IL-12.

Figure 4. Slow IP-10 induction by exogenous IL-12. Non-adherent PBMC were stimulated with 0, 100 and 1000 μg/ml (0, 1 and 10 U/ml of IL-12. IFN-γ (A) and IP-10 (B) were determined from the same experiment.

Figure 5A, B. Stimulation of $CD8^+$ cells with E75 induce significantly higher levels of IP-10 than IFN-γ within 24h in the same experiment. Autologous DC were used as stimulators at stimulator to responder rates of 1:20. E75 was used at 20 μg/ml.

Figure 6.A, B. Rapid and early induction of IP-10 by E75 is only partially inhibited by mAb to CD40L compared with induction of IFN-γ. NP indicates no peptide.

Figure 7 A, B. E75 analogs F42, F43 and F45 induce higher levels of IP-10 than E75. Experimental conditions were as described in the Materials and Methods. E75, F42, F43, and F45 were made at the same concentrations: 25 μl/ml in an autologous HLA-A2 system.

Fig. 8(A) EGF/NDF+VAN induce inhibition of SKBR3.A2 lysis by CTL. This inhibition is partially reversible over time, and by the S/T P-ase inhibitor OA; **(B)** VAN and TPA mediated opposite effects on SKBR3.A2 recognition; **(C)** BB7.2 mAb inhibits recognition of SKBR3.A2

cells suggesting HLA-A2 associated presentation. (A,B,C) Results are from separate experiments.

Fig. 9. Primary CTL induction by TPA + OA treated SKBR3.A2. SKRB3.A2 were treated with EGF + VAN, TPA + OA or TPA + OA and 1 μM of GF109203X as described in the Preliminary Results section and **Fig. 2.** 2 X 10^6 plastic non-adherent PBMC from donor 5 were added at a 20:1 (R:S ratio) in a 24 well plate. IL-2 (50 U/ml) was added after 48 h. CTL activity against C85, or E75 pulsed T2 was determined on Day 5. % specific lysis was obtained by subtracting the % lysis in the presence of peptide from % lysis of T2 in the absence of peptide, which ranged between 8-10%.

Fig. 10 Lane 1: λ DNA - HindIII digest and λX174 - HaeIII digest (M.W. standards). Lanes 2 and 3 show the parental template DNA undigested and digested with EcoRI, respectively. Lanes 4 and 5 show tow wild-type clones of Her2/neu and lanes 6 and 7 show two mutant clones of Her2/neu and lanes 6 and 7 show two mutant clones of Her2/neu with the novel EcoRI DNA fragment. The Y1248 → F mutation was confirmed by sequence analysis. Full length mutant Her2/neu was reconstructed in the original expression vector, pcDNA3.

Conclusions

The research during the previous period made the following contributions to the understanding of immunity in breast cancer and completion of the proposal tasks (1-4). The emphasis of our research was placed on effector functions elicited by recognition of tumor Ag since this is important for therapy.

1. IL-12 is necessary for induction of effector cytokine function in breast cancer patients $CD8^+$ cells when stimulated with a tumor Ag. **Based on this finding plans are made with Dr. Lee Murray Jones to include IL-12 in the vaccination protocols.**

2. Recognition of tumor Ag, from HER-2 and other tumor proteins induce the anti-angiogenic chemokine IP-10. Thus our work identified a novel effector function by CTL recognizing tumor Ag.

3. Recognition of certain Ag variants induce higher levels of cytokines and chemokines than the original (wild-type) Ag. Thus ongoing studies will address and complete the characterization of enhancer Ag agonists.

4. Stimulation of tumor associated lymphocytes from ovarian and breast cancer patients with FBP tumor Ag presented on allogenic DC can induce/enhance specific CTL activity. This finding is important for adoptive therapy in patients with advanced disease where cancer vaccination it is not likely to work.

5. Post-translational modification of the Ag by phosphorylation modulate tumor Ag presentation and their immunogenicity. At least for HER-2 which was investigated Tyrosine phosphorylation inhibited presentation, while serine/threonine phosphorylation apparently enhanced presentation. This raises the possibility of modulating tumor sensitivity with pharmacological activators of Ser/Thr - kinases (protein kinases), or tyrosine phosphatases and raise the possibility of combining drug therapy with immunotherapy.

6. **The use of non-oncogenic (genetically modified) HER-2 can be useful for DNA cancer vaccine development. Its application will not be limited to HLA-A2. Further, the use of**

Ag variants can be used to modulate responses, protect from apoptosis and overcome tollerance.

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APPENDICES

Publications Resulting from this Grant

Anderson, L. D. Jr., Hudson, J. M., Savary, C. A., Fisk, B. Gershenson, D. M., **Ioannides, C. G.** HER-2/neu peptide specificity in the recognition of HLA-A2 by natural killer cells. *Cancer Immunol. Immunother.*, 1999, in press.

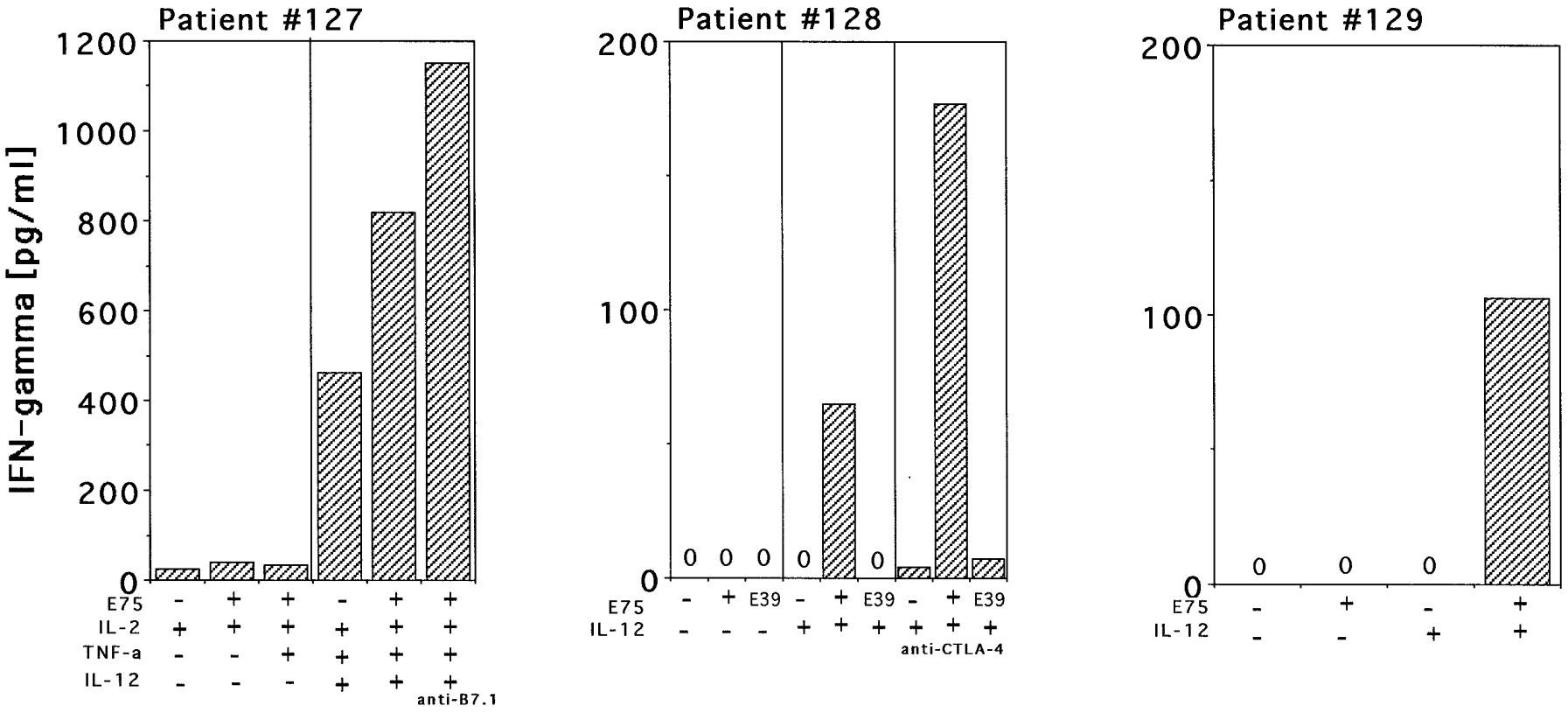
Kim, D. K., Lee T. V., Castilleja, A., Anderson, B. W., Kudelka, A. P., Murray, J. L., Sittisomwong, T., Wharton, J. T., Kim, J. W., **Ioannides, C. G.** Folate binding protein peptide 191-199 presented on dendritic cells can stimulate CTL from ovarian and breast cancer patients. *Anticancer Res., 19: (1999).*

Fisk, B., and **Ioannides, C.G**. Increased sensitivity of Adriamycin-selected tumor lines to CTL mediated lysis results in enhanced drug-sensitivity. *Cancer Res.* 58:4970-4973, 1998.

Babcock, B., Anderson, B.W., Papayannopoulos, I., Castilleja, A., Murray, J.L., Stifani, S., Kudelka, A.P., Wharton, J.T., and **Ioannides, C.G.** Ovarian and breast cytotoxic T lymphocytes can recognize peptides from the amino-enhancer of split protein of the Notch complex. *Mol. Immunol.* 35:1121-1133, 1998.

Peoples, G.E., Anderson, B.W., Fisk, B., Kudelka, A.P., Wharton, J.T., and **Ioannides, C.G.** Ovarian cancer-associated lymphocytes recognize folate binding protein (FBP) peptides. *Ann. Surg. Oncol.* 5(8):743-750, 1998.

Figure 1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1000
800
600
400
200
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IL-2 + + + + + +
TNF-a - - + + + +
IL-12 - - - + + +
anti-B7.1
Patient #128
200
100
0
0 0 0 0 0 0
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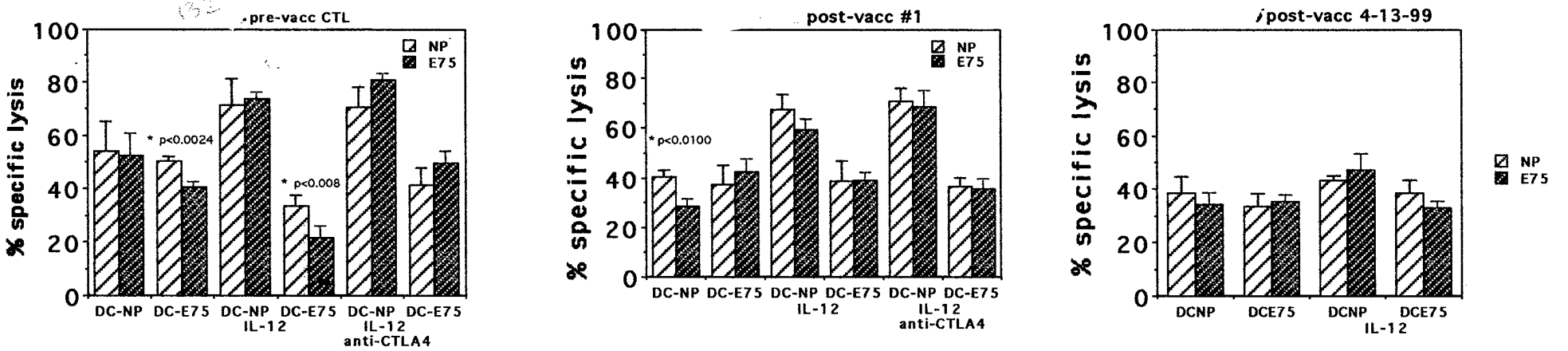
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post-vacc 4-13-99
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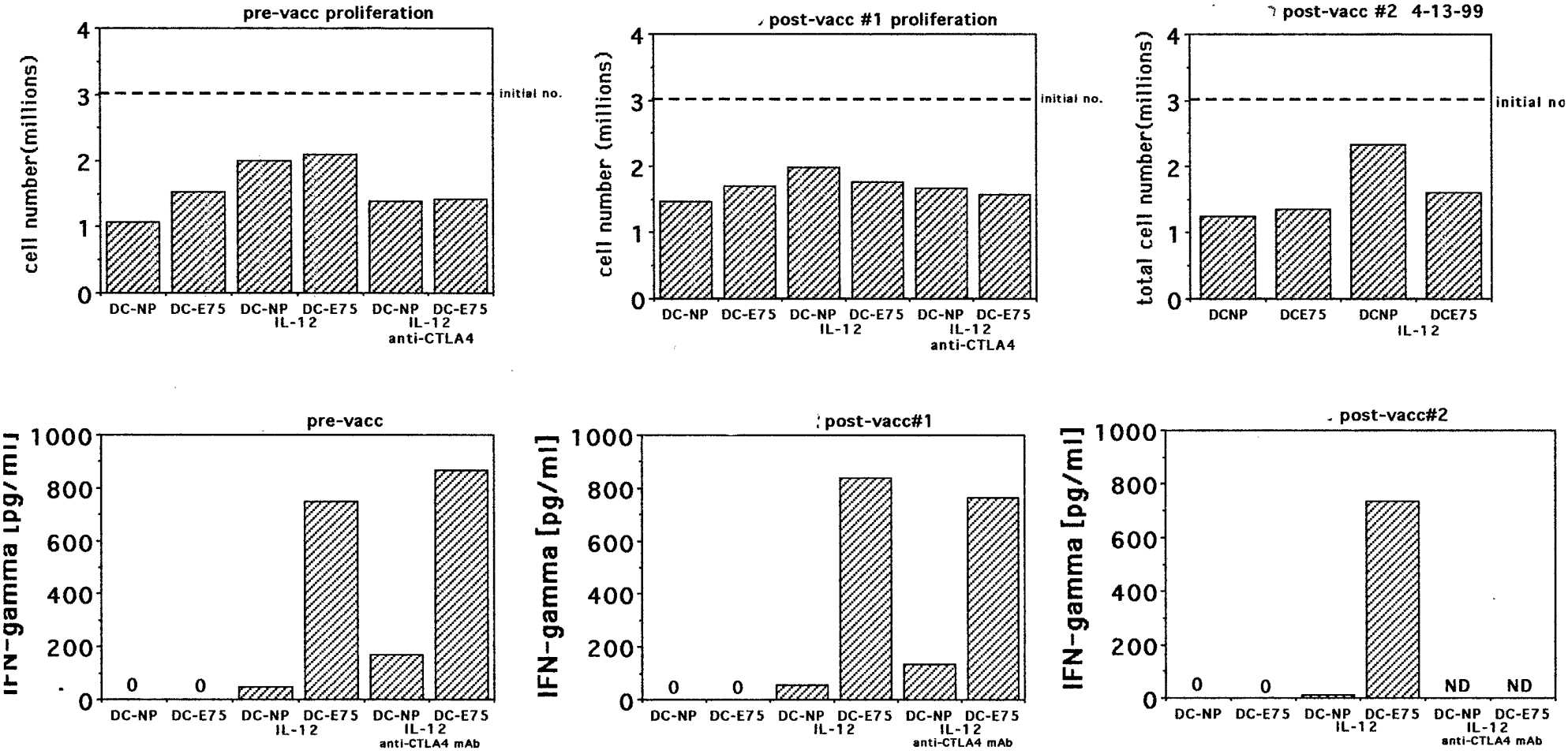
E75
* p<0.0024
* p<0.008
* p<0.0100
DC-NP
DC-E75
DC-NP IL-12
DC-E75 IL-12
DC-NP IL-12 anti-CTLA4
DC-E75 IL-12 anti-CTLA4
DCNP
DCE75
DCNP IL-12
DCE75 IL-12

Figure 2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post-vacc #1 proliferation
post-vacc #2 4-13-99
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total cell number(millions)
initial no.
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DC-E75
DC-NP IL-12
DC-E75 IL-12
DC-NP IL-12 anti-CTLA4
DC-E75 IL-12 anti-CTLA4
DCNP
DCE75
DCNP IL-12
DCE75 IL-12
pre-vacc
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IFN-gamma [pg/ml]
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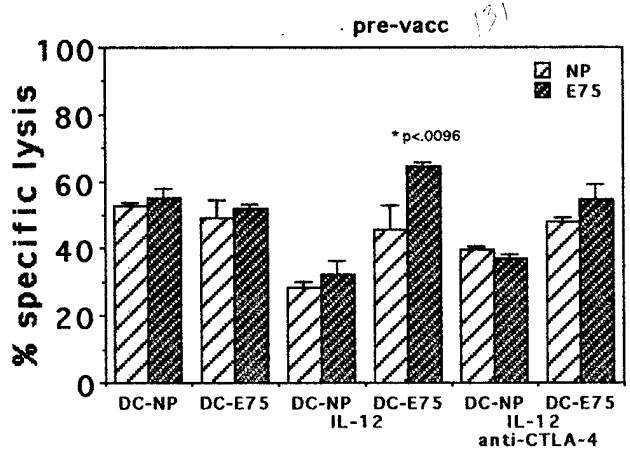
ND
anti-CTLA4 mAb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E75
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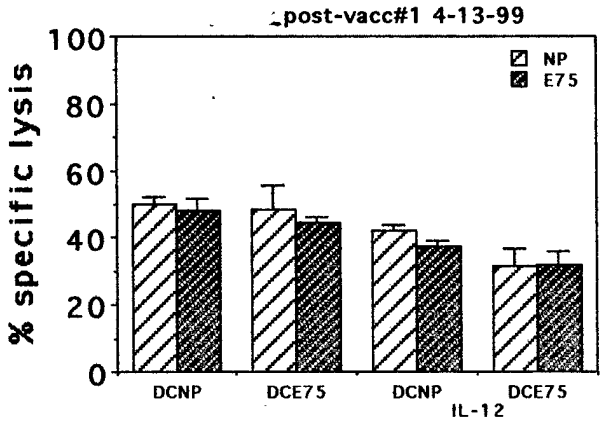
DC-E75 anti-CTLA-4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% specific lysis
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E75
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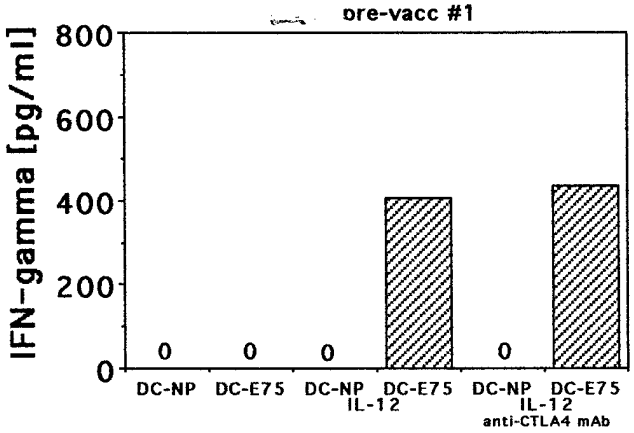
DCNP IL-12
DCE75 IL-12

Figure 3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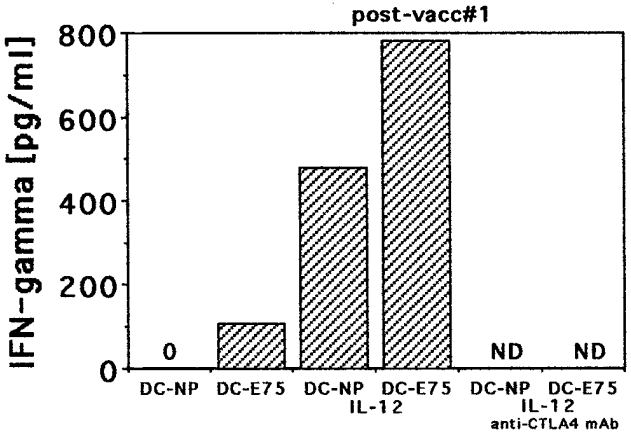
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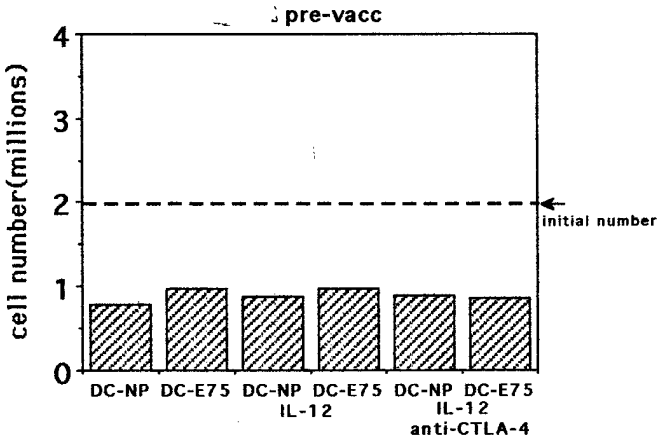
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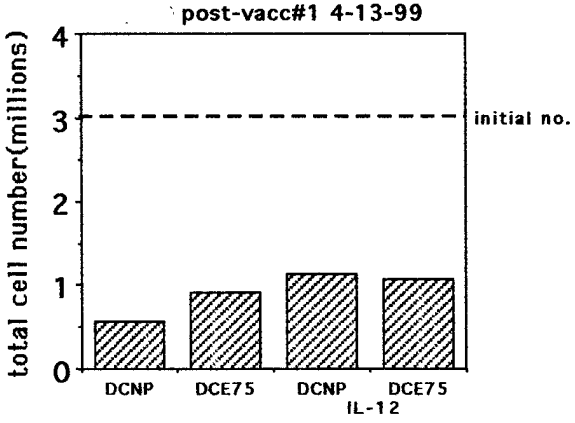
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initial no.
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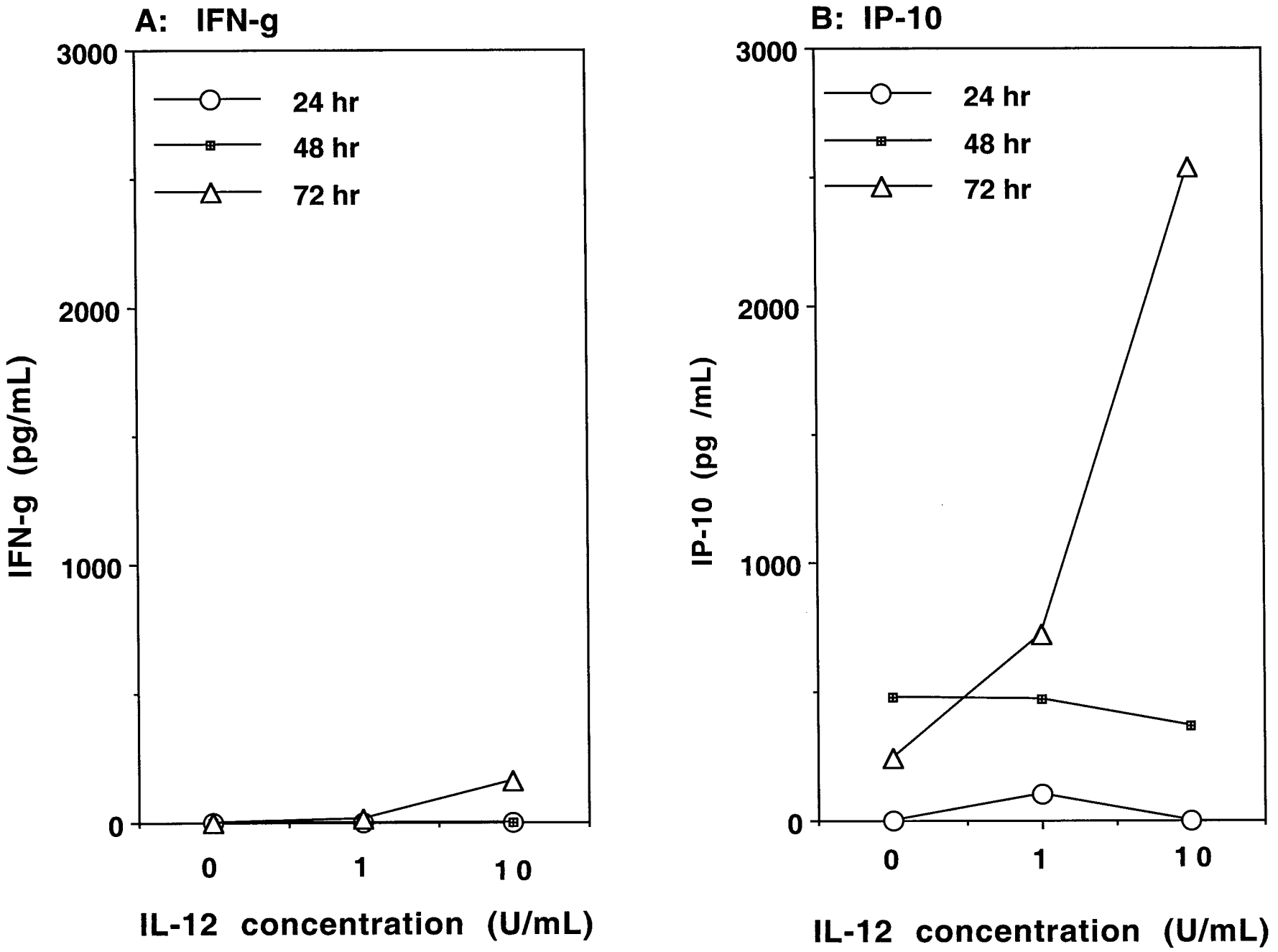
DCNP IL-12
DCE75 IL-12

Figure 4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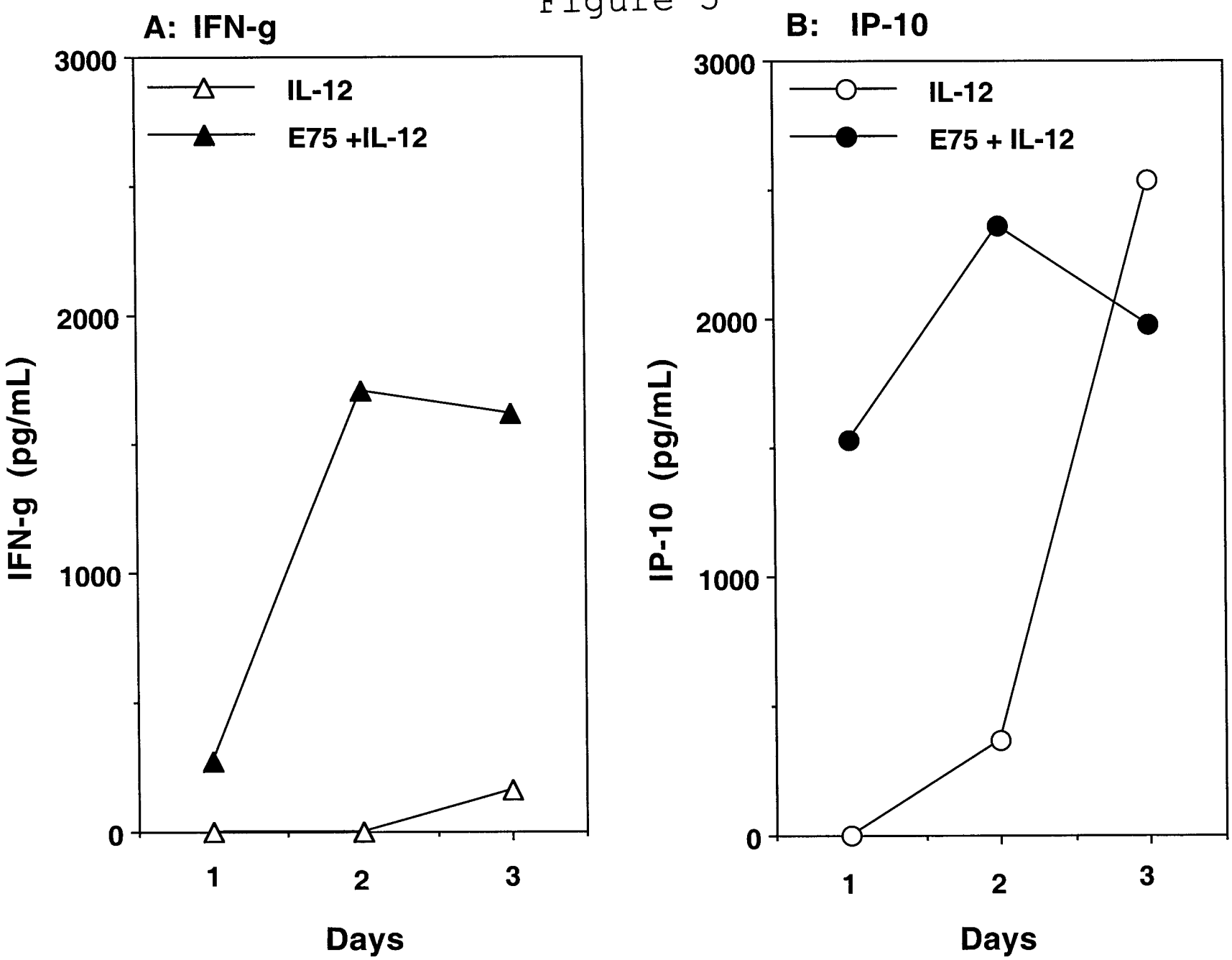
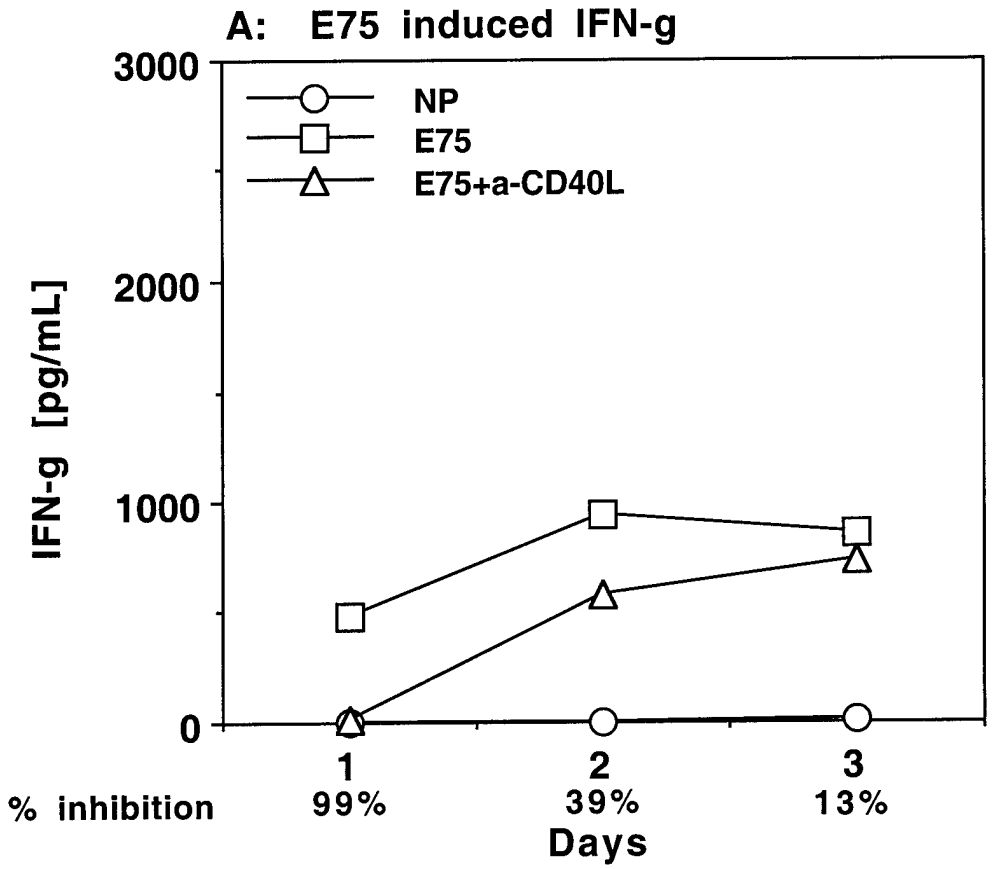
2000
1000
0
24 hr
48 hr
72 hr
IFN-g (pg/mL)
0
1
10
IL-12 concentration (U/mL)
B: IP-10
3000
2000
1000
0
24 hr
48 hr
72 hr
IP-10 (pg /mL)
0
1
10
IL-12 concentration (U/mL)

Figure 5


A: IFN-g
3000
2000
1000
0
IFN-g (pg/mL)
IL-12
E75 +IL-12
1
2
3
Days
B: IP-10
3000
2000
1000
0
IP-10 (pg/mL)
IL-12
E75 + IL-12
1
2
3
Days

α CD40L inhibition of E75 induced IFN-g and IP-10



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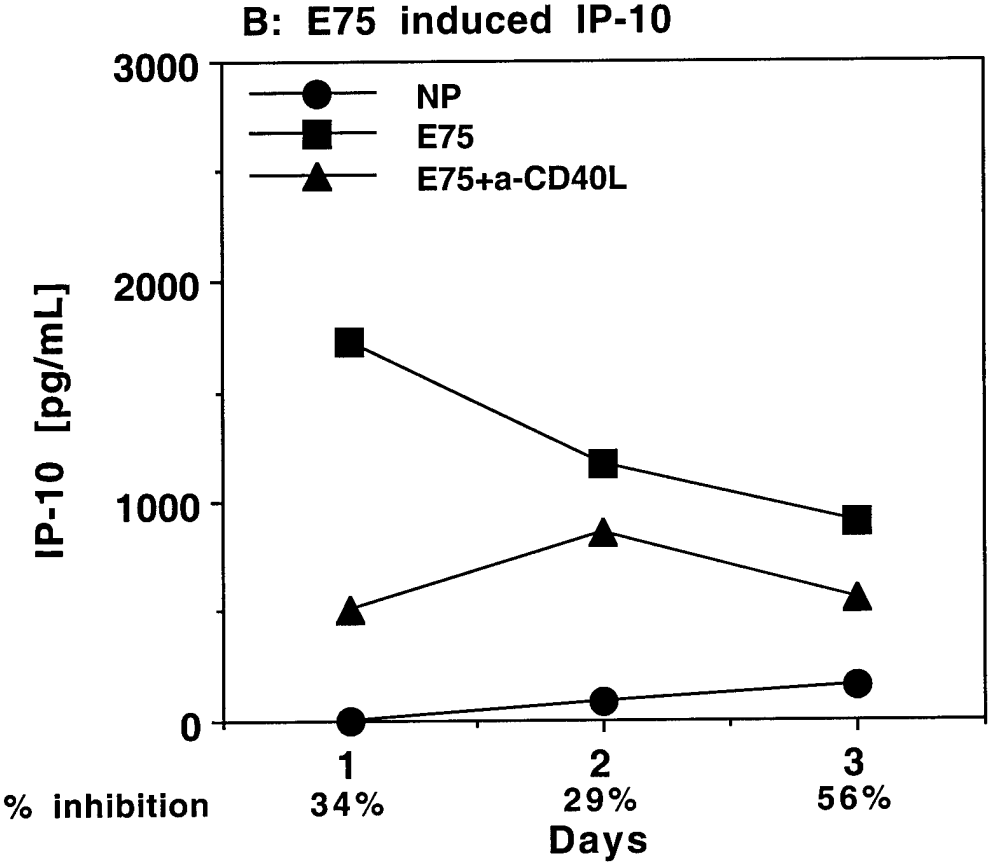

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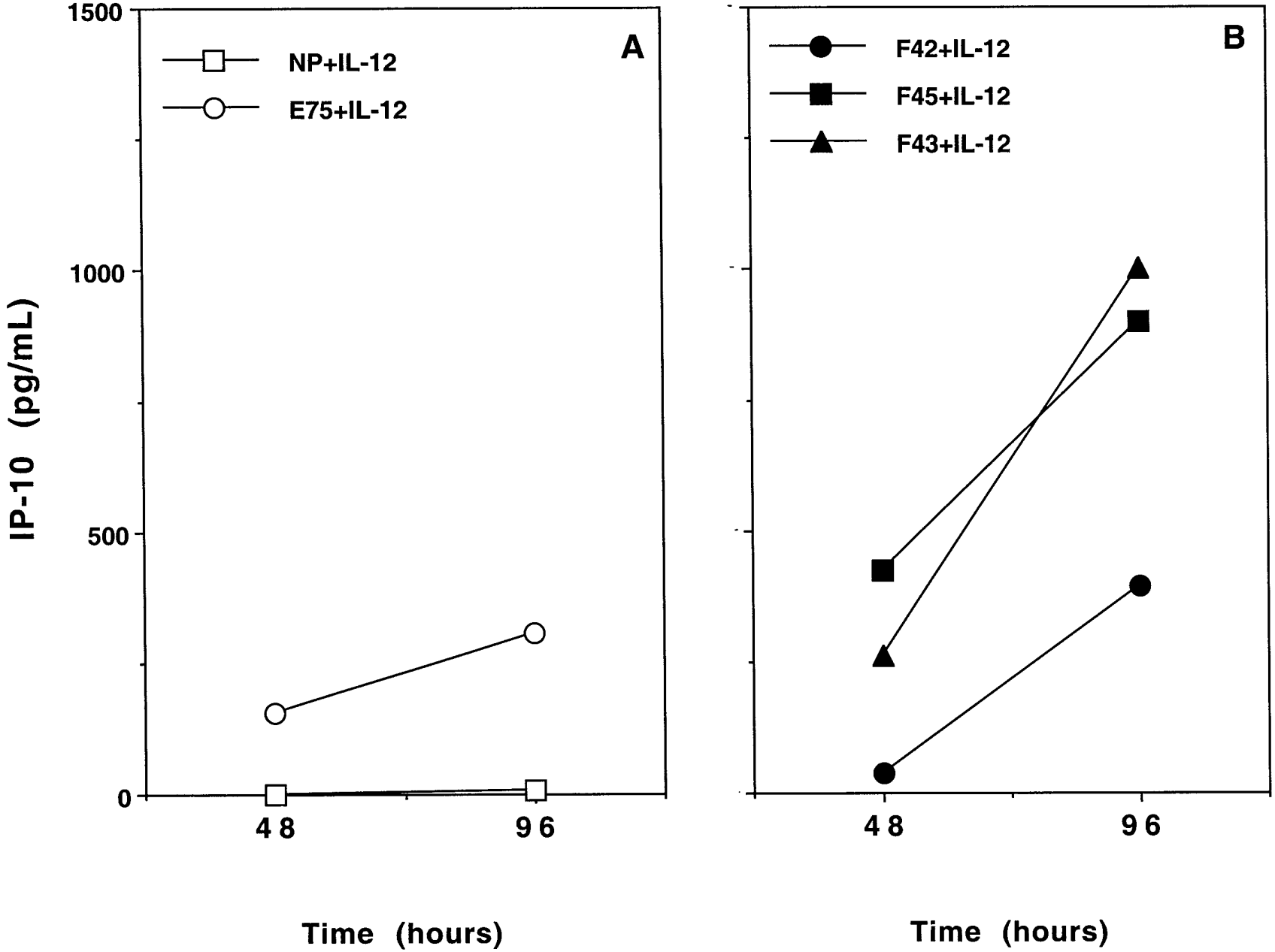
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500
0
IP-10 (pg/mL)
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NP+IL-12
E75+IL-12
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Time (hours)
B
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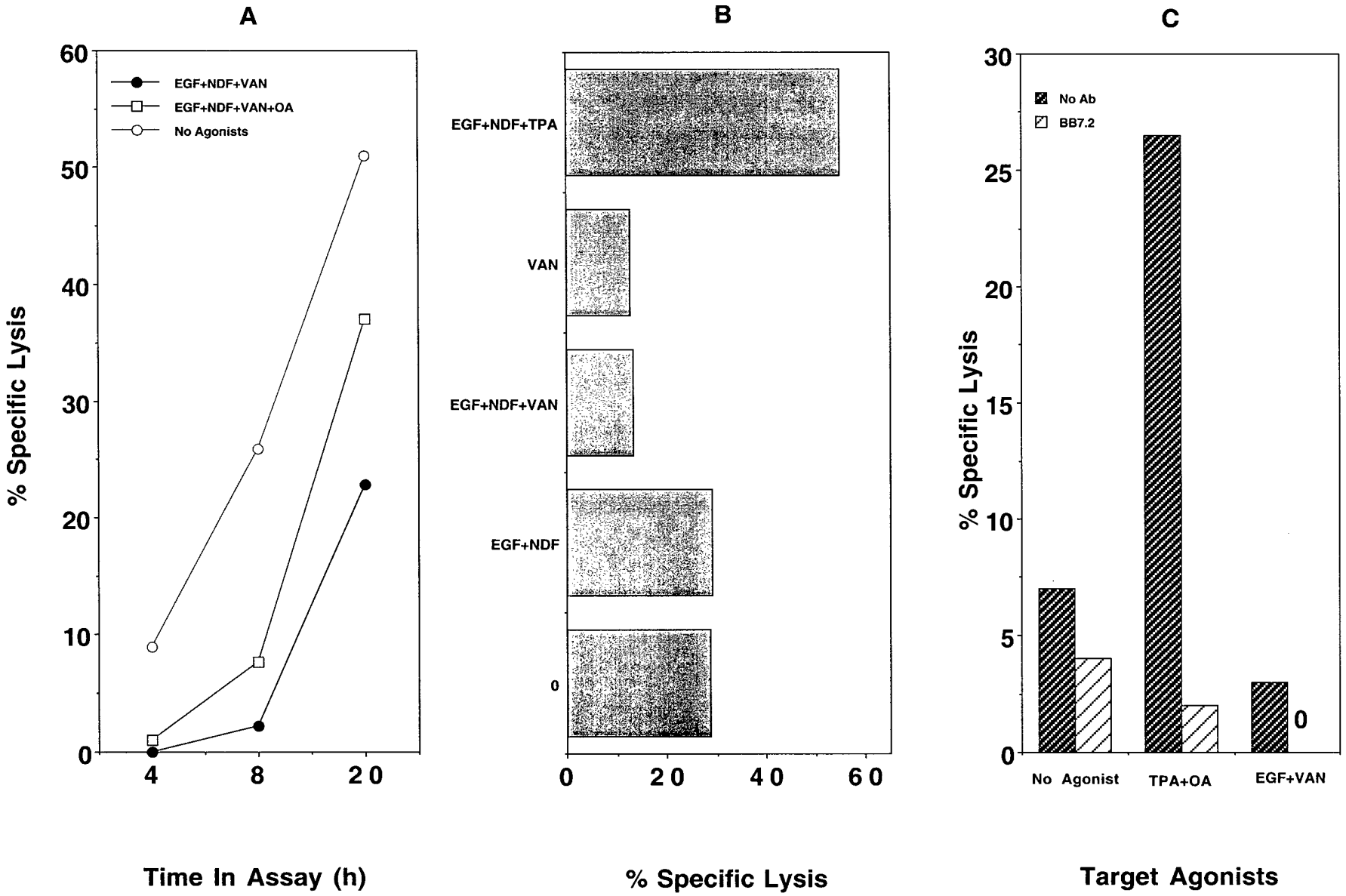
F43+IL-12
48
96
Time (hours)

Figure 8


A
B
C
EGF+NDF+VAN
EGF+NDF+VAN+OA
No Agonists
% Specific Lysis
Time In Assay (h)
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VAN
EGF+NDF+VAN
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BB7.2
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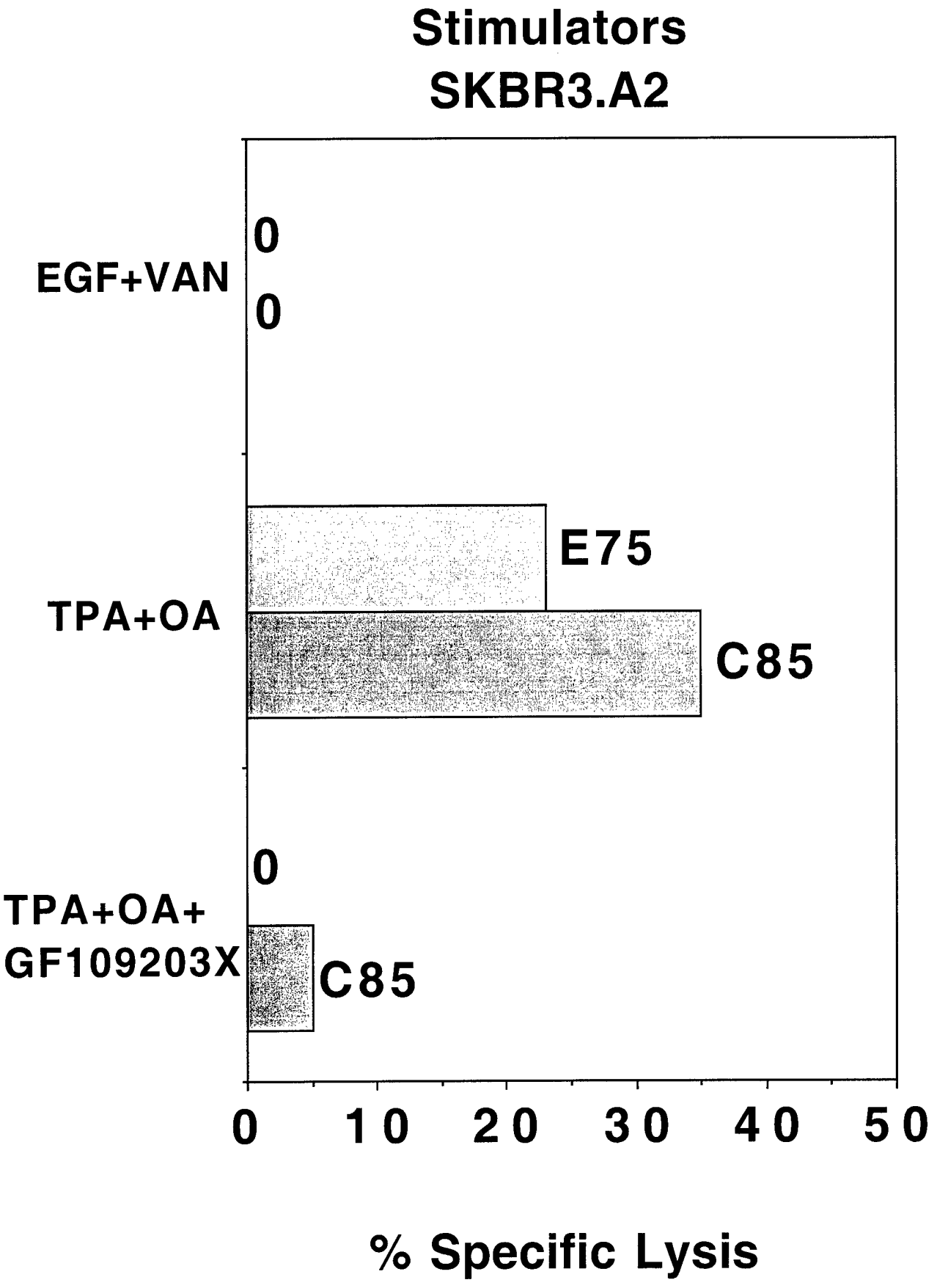
EGF+VAN
Target Agonists

Figure 9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0
C85
0
10
20
30
40
50
% Specific Lysis

[CANCER RESEARCH 58, 4790–4793, November 1, 1998]

Advances in Brief

Increased Sensitivity of Adriamycin-selected Tumor Lines to CTL-mediated Lysis Results in Enhanced Drug Sensitivity[1]

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Departments of Gynecologic Oncology [B. F., C. G. I.] and Immunology [C. G. I.], The University of Texas M. D. Anderson Cancer Center, and The University of Texas Medical School-Houston [B.F.], Houston, Texas 77030

Abstract

The emergence of drug resistance to chemotherapeutic agents is a major cause of treatment failure in cancer therapy. Therefore, much effort has been aimed at circumventing or reversing this undesired effect. Recently, we found that tumor cell lines selected for their multidrug-resistant phenotype can also exhibit increased levels of *TAP* mRNA and MHC class I proteins. This raised the question of whether drug-resistant tumors are more readily recognized by MHC-restricted CTLs. In this report, we show that five of five MHC class I^+ tumor cell lines grown in medium containing Adriamycin developed into variants that expressed higher levels of MHC class I than did their corresponding parental cell lines. This was not observed with a MHC class I^- cell line. No similar association was noted for changes in the expression of either HER-2 or intercellular adhesion molecule 1 protein. We also found that MHC class I^+ drug-selected variants were more readily lysed by MHC-restricted, tumor-associated CTLs than were the drug-sensitive parental cell lines. When the drug-selected variants were cocultured with the same CTLs to eliminate tumor cells expressing higher levels of MHC-I (MHC-I^{hi}), the CTL-resistant tumor cells exhibited a drug sensitivity profile similar to that of the parental cell lines that were not exposed to Adriamycin. These findings suggest that certain chemotherapeutic drugs may increase the immunogenicity of some tumors, and that CTL immunotherapy may help reverse drug resistance.

Introduction

Prolonged exposure of tumor cells to cytotoxic chemotherapeutic drugs such as ADR,[3] etoposides, and *Vinca* alkaloids leads to the development of the MDR phenotype, which results in resistance to various types of drugs (1). The development of multidrug resistance plays a major role in the failure of treatment of many types of cancers. Consequently, much effort has been directed at both understanding its development and deriving the means to reverse or circumvent its effects. Still, this problem represents a major obstacle to progress in cancer therapy.

Two different proteins are known to mediate multidrug resistance activity: (*a*) P-glycoprotein, the product of the *MDR1* gene (2); and (*b*) MRP (3). These proteins are thought to act as energy (ATP)-dependent efflux pumps that prevent the intracellular accumulation of cytotoxic compounds. Both proteins belong to the ABC superfamily of transmembrane transporters, the family that also includes the TAP proteins (4–6). TAP is a heterodimer that transports peptides from the cytosol into the endoplasmic reticulum, where they are available for binding to the MHC class I heavy chain (7). Such presentation of antigenic peptides is a prerequisite for the recognition and lysis of infected or transformed cells by CTLs.

Because of the structural and functional similarities between the genes associated with MDR and TAP, we recently investigated whether MDR tumor cells also have altered peptide transport systems (8). We found that the development of the MDR phenotype was paralleled by an increased accumulation of *TAP* mRNA, resulting in a higher level of MHC class I expression relative to that of the parental cell lines. These findings were recently confirmed by Izquierdo *et al.* (9), who also found both TAP and MHC class I to be overexpressed in several MDR tumors.

The findings of enhanced antigen-presenting capabilities among MDR tumors raised questions about the immune recognition of drug-resistant cells in comparison to their drug-sensitive counterparts. It has been demonstrated in experimental models that anticancer drugs, although often thought of as immunosuppressive, can actually potentiate a variety of immune responses (*e.g.*, delayed-type hypersensitivity and abrogation of tolerance; Ref. 10). One of the most widely studied chemotherapeutic agents in this regard is cyclophosphamide (reviewed in Ref. 10). The immunopotentiation observed with cyclophosphamide is thought to result from the inhibition/depletion of suppressor T cells and may be observed with the administration of cyclophosphamide before tumor challenge (10, 11). It has also been shown that the administration of chemotherapeutic agents such as melphalan can result in increased tumor infiltration by $CD8^+$ T lymphocytes with potent, antigen-specific cytotoxic activity *in vitro* (12). ADR was found to result in a dose-dependent increase in tumor-specific CTL activity in mice receiving tumor cell vaccines, particularly when it was administered 1 week after vaccination as opposed to administration before vaccination (13). Furthermore, the development of regimens that alternate cytotoxic therapy with immunotherapy (sequential chemoimmunotherapy) has demonstrated a synergystic effect of the two modalities in clinical trials (14, 15). The exact mechanism by which chemotherapy induces this immunopotentiation remains to be elucidated. We hypothesized that: (*a*) the increased expression of TAP and MHC class I proteins associated with the MDR phenotype renders such tumor cells more susceptible to recognition and lysis by MHC class I-restricted, tumor-specific CTLs; and (*b*) the elimination of the MHC^{hi} cells within a population of MDR cells results in increased sensitivity of the remaining population of cells to the cytotoxic effects of chemotherapeutic drugs by also eliminating the MDR- or MRP-overexpressing cells.

Materials and Methods

Fluorescence-activated Cell-sorting Analysis. Tumor surface antigens were detected as described previously (16), using an EPICS V Profile Analyzer (Coulter Corp., Hialeah, FL). Antibodies to HLA ABC (W6/32; DAKO, Glostrup, Denmark), HER-2/neu (Ab2; Oncogene Science, Manhasset, NY),

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[2] To whom requests for reprints should be addressed, at Department of Gynecologic Oncology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 67, Houston, TX 77030. Phone: (713) 792-2849; Fax: (713) 792-7586.

[3] The abbreviations used are: ADR, Adriamycin; MDR, multidrug-resistant; TAP, transporter associated with antigen processing; ICAM, intercellular adhesion molecule; ABC, ATP-binding cassette; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IL, interleukin; HLA, human lymphocyte antigen; NFκB, nuclear factor κB.

and ICAM-1 (Calbiochem, San Diego, CA) were not conjugated. Cells to be examined were incubated with the appropriate antibody at 4°C for 30 min, washed, and further incubated with goat antimouse IgG (Boehringer Mannheim, Indianapolis, IN). Cells were washed again after 30 min and then analyzed.

CTL Cytotoxicity Assays. Cytotoxic activity of tumor-associated lymphocytes/CTLs was determined using the *in vitro* ^{51}Cr release assay (16). CTLs used as effectors were generated as described previously (16, 17). For the cytotoxicity assay, 1–2 $\times$ 10^6 target cells were labeled with 100 μCi of ^{51}Cr (Amersham, Arlington Heights, IL) at 37°C for 90 min, washed three times, and plated in triplicate at a final concentration of 5 $\times$ 10^3 cells/well in 96-well V-bottomed microtiter plates (Costar, Cambridge, MA) containing the appropriate number of effector cells. For MHC class I inhibition, 5 μl of W6/32 were added to the appropriate wells. Maximum release was obtained by adding 0.1N HCL. The percentage of specific target cell lysis was determined by the following formula:

$$\frac{\text{Experimental } ^{51}\text{Cr release} - \text{Spontaneous release}}{\text{Maximum } ^{51}\text{Cr release} - \text{Spontaneous release}} \times 100$$

Drug Selection. Drug-selected variants were derived from breast (SKBR3, MCF-7, and MDA MB453) and ovarian (SKOV3, MDA 2774, and CaOV3) tumor cell lines by exposure to gradually increasing concentrations of ADR. In brief, 1 $\times$ 10^6 cells were seeded in T-25 flasks with 12 ml of RPMI-FCS [RPMI 1640 (Life Technologies, Inc.) + 10% FCS + 40 μg/ml gentamicin]. ADR (Sigma) was added at a final concentration of 1 ng/ml. Cultures were split every 3–4 days, at which time the ADR concentration was increased. Concentrations were increased from 1 ng/ml to 2, 4, 10, 15, 20, . . . 100 ng/ml over a 1-month period. ADR-selected tumor cells were 100% viable in 125 ng/ml ADR by the MTT assay. Nonselected parental cell lines were cultured and split simultaneously.

Immunoselection. CTL escape variants were generated from drug-selected tumor cell lines as follows. Cells of the line to be selected were added at 5 $\times$ 10^4 cells/well to a 24-well Falcon plate along with 2.5 $\times$ 10^5 CTLs (CTL-B or CTL-E) in a final volume of 2 ml of RPMI-FCS + 50 units/ml IL-2 (Cetus). A similar number of seeded wells were incubated without IL-2 (RPMI-FCS alone) as controls. After 2 days, the wells were rinsed gently with RPMI 1640, and the nonadherent cells were removed. Both immunoselected and control wells were then further cultured in RPMI-FCS without IL-2 and split as needed. The determination of drug sensitivity was performed 7 days after the initiation of selection with CTLs.

Drug Sensitivity Assay. The assay was modified from that described by Wilson *et al.* (18). Cells were added at 2,5 $\times$ 10^4 cells/well in a final volume of 100 μl of RPMI-FCS to a 96-well flat-bottomed plate, which already contained triplicate dilutions of ADR. Final dilutions of ADR ranged from 4 to 500 ng/ml. Plates were incubated at 37°C for 18 h, after which 25 μl of MTT (at 2.5 mg/ml) was added per well. The plates were incubated for an additional 4 h and then centrifuged for 5 min at 200 $\times$ *g*. Medium and unconverted MTT were removed by inversion, and 75 μl of DMSO were added to each well. Plates were incubated on a rotator for 10 min and then read at 570 nm by a Dynatech auto plate reader. The effect of the cytotoxic drug was determined by calculating the absorbance of the test wells as a percentage of that of the control wells.

Results

ADR-selected Tumor Cell Lines Exhibit Increased Levels of MHC Class I. To examine the effect of chemotherapeutic resistance on tumor MHC class I expression, we exposed six established tumor cell lines (three ovarian and three breast cancer cell lines) to increasing concentrations of ADR. The levels of MHC class I expression of the ADR-selected variants were then determined by fluorescence-activated cell-sorting analysis and compared with those of the corresponding drug-sensitive parental cell lines. Increased levels were found in all five ADR-selected variants that had corresponding MHC class I^+ parental cell lines (Table 1). The levels of increased MHC class I expression varied from a 14% increase observed with SKOV3 to a 156% increase seen with MCF-7. The remaining cell lines exhibited between a 40 and 80% increase in MHC class I expression. One cell line, MDA 2774, consisted of two distinct populations expressing high and low levels of MHC class I. Both populations in the ADR-selected variant, 2774-DR, exhibited increased levels of MHC class I.

Table 1 *Increases in HLA-A, -B, and -C antigen expression in drug-resistant tumor cell lines*

Tumor cell line	Mean level of fluorescence: Drug sensitive	Drug resistant	MCF-R[a]
HLA class I			
MDA 2774	155	215	1.39
SKOV3	190	216	1.14
CaOV3	194	346	1.78
MCF-7	80	205	2.56
SKBR3	80	120	1.50
MDA MB453	44[b]	44[b]	1.0
HER-2/neu			
MDA 2774	153	153	1.0
SKOV3	385	396	1.0
CaOV3	10	10	1.0
MCF-7	335	320	0.95
SKBR3	139	139	1.0
MDA MB453	613	536	0.87
ICAM-1			
MDA 2774	230	190	0.82
SKOV3	111[b]	119[b]	1.27
CaOV3	78	99	1.27
MCF-7	275	274	1.0
SKBR3	50	60	1.0
MDA MB453	116	119[a]	1.0

[a] MCF-R, mean channel fluorescence ratio was obtained by dividing each value in the second column by the corresponding value in the first column (*i.e.*, 215:122 = 1.39).
[b] Negative samples (antibody-stained population showed no difference in comparison to the negative control).

One parental tumor cell line, MDA MB453, which was negative for MHC class I expression, was used as a control. The corresponding ADR-selected variant, MB453-DR, was the only drug-selected cell line that did not show any changes in MHC class I expression. Hence, the loss of MHC class I expression is not corrected by selection with ADR or the development of drug resistance.

Increased expression of the proto-oncogene HER-2 has also been described as being associated with MDR1-overexpressing breast and ovarian tumors (19). Because increased HER-2 expression results in CTL recognition (17), we also determined the levels of HER-2 expression on the ADR-selected variants. As shown in Table 1, HER-2 expression was slightly increased in one cell line, unchanged in three cell lines, and decreased in two other cell lines. We also examined for differences in ICAM-1 expression between drug-selected and nonselected tumor cells, because this adhesion molecule can facilitate tumor recognition by cellular immune effectors. Two of the six tumor cell lines were negative for ICAM-1 expression, as were the corresponding drug-selected variants. Of the four ICAM-1-positive cell lines, two showed an increase in ICAM-1 expression in the drug-selected variants, one showed no change, and one showed a slight decrease. Thus, whereas MHC class I expression was clearly increased in all ADR-selected variants, HER-2 and ICAM-1 expression showed no such association.

Increased CTL-mediated Lysis of ADR-selected Tumor Cell Lines. CTL cytotoxicity assays were performed to determine whether the increased levels of MHC class I expression in ADR-selected variants resulted in increased target sensitivity to lysis. To ensure that the results were relevant for several HLA types, we used three ovarian CTL lines that express at least one HLA in common with the MDA 2774 cell line (CTL-B, HLA-A3; CTL-E and CTL-R, HLA-A24) as effectors. These CTL cell lines have been previously shown to preferentially lyse autologous tumors (15). All three CTL cell lines lysed

the parental MDA 2774 cells, but a higher lysis of the ADR-selected 2774-DR variant than of the parental cell line was observed (Fig. 1, *A* and *B*). Lysis was inhibited by the addition of the anti-MHC class I antibody W6/32 (Fig. 1*B*). As expected, neither the MHC class I^- MDA MB453 nor the MB453-DR tumors were lysed, indicating that the increased sensitivity to lysis is dependent on MHC class I expression, and that tumor lysis by these effectors is not likely to be the result of a natural killer-lymphokine-activated killer cell activity.

A fourth CTL cell line, CTL-V (HLA-11, B60, 62), was tested against SKBR3 (HLA-A11, B18, 40) and its ADR-selected variant, SKBR3-DR. Neither SKBR3 nor SKBR3-DR was lysed by CTL-V (data not shown). Because both targets shared HLA-A11 but expressed lower levels of MHC class I than did the other tumor cell lines tested (Table 1), we retested CTL-V-mediated lysis after pretreating the tumors with 300 units/ml IFN-γ for 24 h. However, the IFN-γ-treated tumors were still resistant to lysis (data not shown). These results suggest that CTLs that lack antigen recognition of the ADR-sensitive tumor will not recognize the ADR-selected tumor. Drug selection did not appear to alter the antigen profile of the tumor as recognized by these effectors but merely increased the antigen presentation.

Immunoselection with CTLs of ADR-selected Variants Increases Drug Sensitivity. On the basis of findings of increased sensitivity of ADR-selected variants to CTL-mediated lysis, we hypothesized that selection by the CTLs may result in the elimination of those tumor cells with greater drug resistance potential. If this hypothesis is correct, then the resulting population of CTL escape tumor variants would then be more susceptible to the cytotoxic activity of ADR. To test this hypothesis, we derived CTL escape variants by coculturing the drug-selected cell lines with CTLs. We then compared the ADR sensitivity of the CTL escape variants with that of both the non-CTL-selected drug-resistant variants (cultured for the same interval in the absence of ADR) and the drug-sensitive parental cell lines in MTT assays. As shown in Fig. 2*A*, the non-CTL-selected 2774-DR cell line was resistant to the cytotoxic activity of ADR up to concentrations of 125 ng/ml. The parental MDA 2774 cell line exhibited sensitivity at ADR concentrations as low as 8 ng/ml. Interestingly, the CTL-resistant variant derived from 2774-DR by selection with CTL-B exhibited an ADR sensitivity profile that was indistinguishable from that of the parental MDA 2774 cell line.

We repeated the experiment with CTL-E and the SKOV3-DR cell line, which share HLA-B35. Based on the MTT assay, the parental SKOV3 cell line appears to be more inherently resistant to ADR than MDA 2774, exhibiting sensitivity only at high concentrations of ADR ($\geq$250 ng/ml; results not shown), similar to the profile of the drug-selected SKOV3-DR cell line. Thus, the pattern of sensitivity to ADR was in the range of 4–125 ng/ml (Fig. 2*B*). This may be a reflection of prior *in vivo* selection with chemotherapeutic drugs, which is supported by the observation that among the MHC class I^+ cell lines, SKVO3 exhibited the lowest increase in MHC class I expression with ADR exposure (Table 1). Of interest, the CTL-resistant variant of SKOV3-DR was sensitive to much lower concentrations of ADR than were the SKOV3 and SKOV3-DR cell lines. Thus, our findings suggest that CTL-mediated lysis could eliminate those cells within a tumor population that are more resistant to ADR, leaving a more susceptible population.

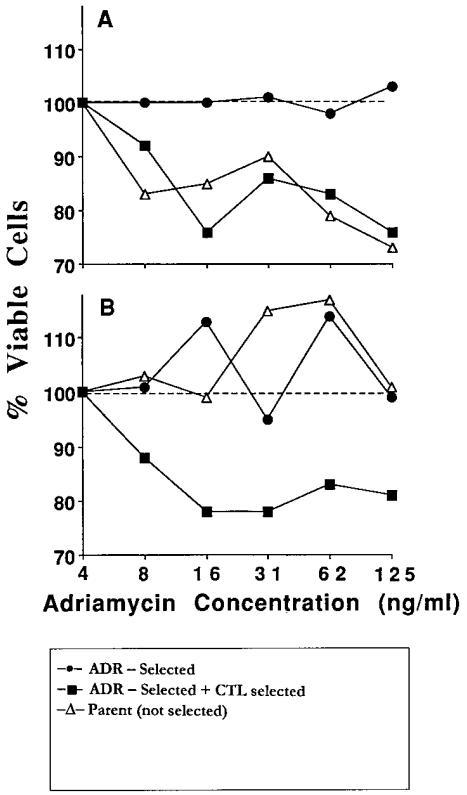

Fig. 2. Increased drug-sensitivity in CTL-resistant variants of the ADR-selected lines. ADR-selected MDA 2774 (*A*) and SKOV3 (*B*) cells were cultured in the presence (■) or absence (●) of HLA-matched CTLs (CTL-B and CTL-E, respectively) for 7 days. Parental cell lines without previous ADR exposure (△) were cultured under identical conditions as the nonimmunoselected drug-selected cell lines. The subsequently derived tumor cell lines were examined in drug sensitivity assays as described in "Materials and Methods."

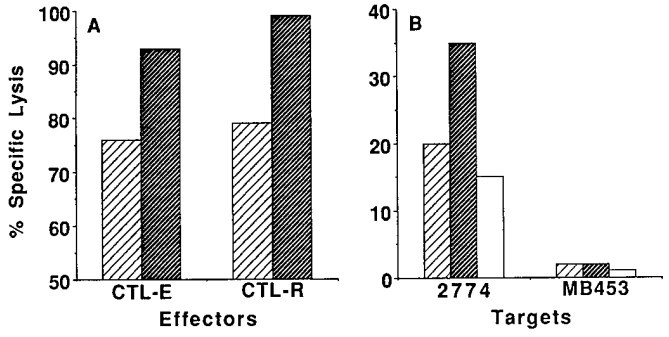

Fig. 1. Increased susceptibility of the drug-selected MDA 2774 tumor cell line to CTL-mediated lysis. CTL-E and CTL-R (*A*) and CTL-B (*B*) were tested for lysis of unselected (▨) and ADR-selected (▨) 2774 tumor cell lines. Additionally, CTL-B was tested against the ADR-selected MDA 2774 cell line in the presence of anti-MHC class I antibody (□) as well as against the MHC class I^- cell line MDA MB453 (*B*).

Discussion

In this report, we present novel evidence that the development of resistance to chemotherapeutic agents such as ADR is associated with the increased susceptibility of tumors to CTL lysis. This is paralleled by an increase in MHC class I expression. Increased levels of MHC class I associated with drug selection resulted in an increased sensitivity to CTL-mediated lysis. Lysis was MHC restricted and required MHC expression. Lysis also required the tumor to present peptide antigen to be recognized by TCR. This was suggested by the finding that CTL-V, which could not lyse the MHC class I^+ SKBR3 parental cell line, was also unable to lyse the drug-resistant SKBR3-DR cell line, even after pretreatment with IFN-γ. Therefore, the increased sensitivity to lysis of drug-resistant variants seems to require both an intact antigen presentation pathway and the presence of antigen recognized by effectors.

Immunoselection by coculture of drug-selected tumors with CTLs resulted in the reversion of the surviving tumor cells to a more

drug-sensitive status. However, this did not reflect a spontaneous reversion of escaping tumors to a sensitive phenotype, because the same cells cultured in the same conditions without CTLs were far more resistant to ADR. Thus, the induction of reversion of drug sensitivity is likely the result of the elimination of those tumors expressing the MDR phenotype with a concomitantly higher level of MHC class I. If exposure to a chemotherapeutic drug selects for tumor cells with increased expression of both the MDR phenotype and MHC class I, it follows that after the elimination of those cells, the remaining population of cells will have a lower potential for expressing the MDR phenotype. The mechanisms involved in the increased TAP/MHC class I expression are not known but may involve common transcription factors and intermediates (adapter proteins) also used by the proteins encoded by the drug resistance genes MDR and MRP. NFκB has recently been demonstrated to be involved in the transcriptional regulation of genes in the *mdr* family (19) as well as the regulation of TAP1 expression (20). NFκB activity can be induced by a variety of stimuli, including cytotoxic compounds and other cellular stressors (21). The concomitant induction of stress response NFκB transcription factors in response to cytotoxic stress may therefore play a role in the increased expression of both *mdr* and TAP. Moreover, the Raf-1 kinase, which activates NFκB, was also found to be involved in *mdr* expression (21, 22).

It is important to emphasize that MHC class I expression does not appear to be required for the development of the drug-resistant phenotype induced by ADR. The absence of a requirement for MHC class I is demonstrated by the ability to select the MHC class I^- cell line MDA MB453 in ADR. The drug-selected variant was MHC class I^- as well. Thus, genetic changes resulting in the development of drug resistance do not seem to result in changes that can compensate for existing genetic defects in MHC class I expression. We also did not find increased expression of HER-2 and ICAM-1, making it unlikely that these play a role in the increased lysis of drug-selected tumors by CTLs.

Together, our findings suggest a possible mechanism for synergy between chemotherapeutic agents and immunotherapy. Chemotherapy may decrease the tumor burden, but the remaining tumor cells, as shown here, will express increased levels of MHC class I. These remaining cells are then more likely to be recognized and eliminated by tumor-specific CTLs. Furthermore, CTLs may then eliminate those tumor cells with a higher potential for chemotherapeutic resistance, thus sensitizing the tumor population for a subsequent round of chemotherapeutic drug exposure. Repeated rounds of sequential chemoimmunotherapy may thus result in enhanced responses through greater reductions in tumor burdens.

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Ovarian Cancer-Associated Lymphocyte Recognition of Folate Binding Protein Peptides

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Background: Tumor-associated lymphocytes (TAL) isolated from ovarian cancer patients contain cytotoxic T lymphocytes (CTL) capable of recognizing specific HLA/peptide complexes on tumor cells leading to tumor cell lysis. Currently, HER2/neu, overexpressed in only 30% of breast and ovarian cancers, is the only known source of CTL-recognized peptides in epithelial cancers. Therefore, we have investigated peptides derived from folate binding protein (FBP), which is over-expressed in more than 90% of ovarian cancers and in the majority of other epithelial tumors.

Methods: TAL were isolated from the malignant ascites of four consecutive HLA-A2$^+$ ovarian cancer patients and incubated in IL-2. Initial chromium-release assays were performed within 1 week. T2 cells, incubated with peptide, were used to reconstitute T cell epitopes. The FBP sequence was interrogated for HLA-A2 binding peptides, and five were synthesized (E37–41).

Results: Freshly cultured, unstimulated ovarian TAL recognize peptides derived from FBP. These peptides are presented in the context of HLA-A2, and are specifically recognized in a HLA class I–restricted fashion. TAL recognition of these reconstituted T cell epitopes is concentration dependent. Furthermore, the FBP peptides are shown by cold target inhibition studies to be naturally processed and presented antigens.

Conclusions: FBP peptides are recognized by freshly isolated TAL from ovarian cancer patients, suggesting in vivo expression and sensitization. Because FBP is over-expressed 20-fold in most adenocarcinomas, these peptides may be used in a widely applicable peptide-based vaccine for epithelial tumors.

Key Words: CTL—Peptide—Vaccine—Folate binding protein

A specific anticancer immune response has been well established in melanoma and, to a lesser degree, in epithelial tumors. However, in cancers of the ovary, breast, lung, and pancreas, tumor-specific cytotoxic T lymphocytes (CTL) have been isolated from tumors, suggesting a host response.[1–4] The most intensely studied epithelial tumor is ovarian cancer.[5–9] This tumor has provided an invaluable model for the study of the specifics and similarities of the immune response to epithelial cancers as compared to melanoma. Ovarian cancer, which is the fourth leading cause of cancer death among American women,[10] offers some unique advantages as a tumor model for immunologic research. This disease often presents in advanced stages with bulky disease and malignant ascites, and the primary treatment involves a staging laparotomy with tumor debulking, resulting in large volumes of solid and ascitic tumor for laboratory use. Tumor-reactive CTL are readily and reproducibly isolated from both the solid tumors and ascites, and the latter can be recollected multiple times without surgery.[1,5–9] The motivation for developing immunologic alternative therapies for this disease is high, because no effective treatments currently exist for women who fail primary platinum or taxol-based chemotherapy.[10]

The classic interaction between the T cell receptor (TCR) on the CTL and the HLA/peptide complex on the tumor cell has been verified in the ovarian model, and as in melanoma, HLA-A2, which is expressed in 50% of Caucasians, has been confirmed as a restriction ele-

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ment.[11] However, the most promising aspect of ovarian cancer research to date has been the discovery that common CTL-recognized, tumor-associated antigens (TAA) are expressed not only on various ovarian cancers, but also on multiple other epithelial tumors. For example, we have shown that ovarian cancer-specific CTL also recognize common determinants on HLA-matched colon and pancreas cancers.[12] Likewise, in separate studies, we have found that tumor-specific CTL isolated from HLA-matched ovarian and breast cancer patients are cross-reactive for tumor recognition.[13]

In searching for these common TAA, we also have demonstrated the concept of shared TAA among various epithelial tumors by acid elution studies. Using an ovarian cancer cell line[14] and freshly isolated tumor cells,[15] we have eluted the peptide antigens out of the HLA molecules, fractionated them, and reconstituted these epitopes on the HLA-$A2^+$ antigen processing defective mutant T2.[16] We have shown that ovarian, breast, and non–small cell lung cancer-specific CTL recognize the same antigenic fractions, further proving that common TAA exists among epithelial-derived tumors.[14]

The identification of these TAA has progressed slowly despite the rapid developments in vaccine research in melanoma; however, one such antigen system has been found and confirmed by different groups. HER2/neu is a proto-oncogene, and its protein product has been shown to be the source of multiple peptides that are recognized by ovarian, breast, pancreas, and lung cancer-specific CTL.[3,4,13,17,18] Fortunately, HER2/neu expression has been demonstrated in multiple epithelial-derived tumors.[19,20] However, because this is a normal, nonmutated protein, its usefulness as an immunologic target depends on its level of overexpression, and HER2/neu is overexpressed in only 30% of ovarian and breast cancers.[21]

Folate binding protein (FBP), also known as LK26 trophoblast antigen[22] and GP38,[23] is a membrane-associated glycoprotein recognized by the monoclonal antibodies (mAb) LK26, MOv18, and MOv19 and found to be overexpressed in the vast majority of ovarian cancers.[22,24] The level of expression has been found to be more than 20-fold higher in malignant cells than in normal cells,[25] and in one study was reported to be 80- to 90-fold higher.[24] FBP has been the focus of many studies using mAbs,[26,27] folate conjugates,[28,29] and antifolates,[30] but has not yet been investigated as a potential source of CTL-recognized peptides for use in anti-cancer vaccines.

In this study, we demonstrate that fresh tumor-associated lymphocytes (TAL) from consecutive ovarian cancer patients recognize FBP peptides in an HLA-A2–restricted fashion, and that these peptides are naturally processed antigens.

MATERIALS AND METHODS

Tumor-Associated Lymphocyte Cultures

TAL were isolated from fresh collections of malignant ascites obtained through the Department of Gynecologic Oncology at The University of Texas M. D. Anderson Cancer Center under the approval of the Institutional Review Board. Specimens were processed as previously described.[5] Briefly, malignant ascites was collected sterilely in heparinized containers and immediately transported to the laboratory. The cellular elements of the ascites were obtained by centrifugation and washed with serum-free RPMI-1640. Once resuspended, the lymphocytes and tumor cells were separated by centrifugation over discontinuous 75%/100% Ficoll-Histopaque (Sigma, St. Louis, MO) gradients. Freshly isolated TAL were suspended in RPMI-1640 containing 100 μg/mL L-glutamine (Gibco, Grand Island, NY) supplemented with 10% FCS (Sigma), 40 μg/mL gentamicin, and 50 to 100 IU/mL IL-2 (Cetus, Emeryville, CA). T cells were cultured at 0.5 to 1.0 × 10^6 cells/mL and placed in a humidified incubator at 37°C in 5% CO_2 and maintained at this concentration with the addition of media as needed and IL-2 every 2 to 3 days, depending on the growth kinetics. Consecutive specimens were processed and cultured.

Tumor Targets

The SKOv3 ovarian cancer cell line (ATCC, Rockville, MD) was transfected with the HLA-A2 expression vector RSV.5-neo with resulting high levels of HLA-A2 expression, as previously described.[31] This cell line is maintained in RPMI-1640 with 10% FCS and 250 μg/mL G418 (Sigma). Fresh-frozen tumor was collected from the malignant ascites after Ficoll separation and frozen in aliquots in liquid nitrogen until used.

Phenotype Analysis

The HLA-A2 status of these TAL lines was determined by indirect staining with anti–HLA-A2 mAbs BB7.2 and MA2.1 (ATCC), 50 μL of 1 : 50 dilution of culture supernatant at 4°C for 30 minutes, followed by a 30-minute incubation with goat antimouse mAb conjugated with FITC (Becton Dickinson, Mountain View, CA) and analyzed on a Coulter Epics C Cytometer (Coulter Electronics, Hileah, FL).

Synthetic Peptides

Peptides were synthesized in the Synthetic Antigen Laboratory of The University of Texas M. D. Anderson Cancer Center using solid phase techniques on an Applied Biosystems 430 peptide synthesizer (Applied Biosystems, Foster City, CA). Crude products were dis-

solved and injected onto C-18 4.6-mm I.D. reverse phase HPLC columns (Rainin) and eluted with linear TFA-acetonitrile gradients. Identity and purity of final materials were established by amino acid analysis and analytical RP-HPLC. All peptides used in this study were between 92% and 95% pure. All peptides were derived from the FBP sequence and contained I/L/V at the dominant anchor sites, P2 and P9, necessary for HLA-A2 binding.

HLA-A2 Stabilization Assays

Indirect assessment of peptide binding was performed by HLA-A2 stabilization assays, as previously described.[32] Briefly, T2 cells were pulsed overnight with saturating quantities (100 μg/mL) of each peptide. The cells were then washed and FACS analysis performed as above with BB7.2 and confirmed with MA2.1. HLA-A2 expression was then quantitated as mean channel fluorescence and compared to the expression level on non–peptide-loaded T2. Stabilization is expressed as a ratio of the HLA-A2 expression of peptide-loaded T2 to unloaded T2.

Cytotoxicity Assays

Cytotoxicity was determined by standard chromium release assays, as previously described.[5] Briefly, targets were labeled with 100 to 150 μCi of sodium chromate (Amersham, Arlington Heights, IL) for 1.5 hours at 37°C, then washed twice and plated at 2000 to 2500 cells/well in 100 μL in 96-well round-bottom plates (Costar, Cambridge, MA). Effectors were added at designated effector : target (E : T) ratios in 100 μL per well. After 5 to 20 hours of incubation, 100 μL of culture supernatant was collected, and radionuclide release was measured on a gamma counter (Gamma 5500B, Beckman, Fullerton, CA). All determinants were done in triplicate. Results are expressed as percent-specific lysis as determined by the following formula:

$$(\text{experimental mean cpm} - \text{spontaneous mean cpm}) / (\text{maximum mean cpm} - \text{spontaneous mean cpm}) \times 100.$$

Peptide-Pulsed Cytotoxicity Assays

For these experiments, the T2 cell line (generously donated by P. Creswell) was used. This cell line is a human T cell/B cell fusion product containing an antigen-processing defect in the TAP proteins such that HLA-A2 molecules are empty on the cell surface or contain relatively few bound peptides that can be effectively displaced by exogenous HLA-A2–binding peptides.[16] The T2 cells were labeled with chromium as above, washed, and then incubated with peptide for 1.5 hours at 37°C before standard cytotoxicity assays were performed.

mAb-Blocking Assays

Before the standard cytotoxicity assays were performed, peptide-pulsed T2 was incubated with anti–HLA-A2 mAb BB7.2 (50 μL of 1 : 50 dilution of culture supernatant/well) or anti–HLA-A,B,C mAb W6/32 (ATCC) (5 μL/well) for 30 minutes at 37°C before the effectors were added.

Cold Target Inhibition Assays

Unlabeled T2 was incubated with peptide for 1.5 hours, then added to standard cytotoxicity assays with chromium-labeled tumor targets and effectors. The cold : hot target ratio was 15 : 1. T2 without peptide also was used as a control.

Cold Target Inhibition Assays

Unlabeled T2 was incubated with peptide for 1.5 hours, then added to standard cytotoxicity assays with chromium-labeled tumor targets and effectors. The cold : hot target ratio was 15 : 1. T2 without peptide also was used as a control.

RESULTS

Folate Binding Protein–Derived Peptides

The FBP sequence was interrogated for potential HLA-A2–binding nonamers utilizing the known binding motifs for this molecule.[8] Five peptides were selected for synthesis, based on the presence of leucine, isoleucine, or valine in the dominant anchor positions P2 and P9, and the potential of these peptides to form amphiphilic structures.[8] Peptides were selected with a wide range of predicted binding affinity. An indirect analysis of HLA-A2 binding was performed with HLA-A2 stabilization assays, which are based on the concept that peptide affinity is directly proportional to enhanced HLA-A2 expression because peptides stabilize the HLA molecules on the cell surface of T2. Therefore, a peptide with high affinity causes more HLA stabilization and higher expression. The peptide sequences, their positions, and their relative binding affinities are listed in Table 1. The HLA-A2 expression is given as a ratio of peptide-induced expression over expression on unloaded T2. Four of the peptides are relatively low-affinity binders, whereas E38 is a high-affinity binder.

FBP Peptide Recognition by Ovarian Cancer–Associated Lymphocytes

Four consecutive ovarian malignant ascites specimens, which were subsequently found to be HLA-A2 positive, were processed, and the TAL isolated and cultured in

TABLE 1. *FBP-derived peptide sequences and HLA-A2 stabilization assays to confirm peptide binding and relative affinities*

FBP peptide	Sequence	HLA-A2 (MCF ratio)*
E37 (25–33)	RIAWARTEL	1.16
E38 (112–120)	NLGPWIQQV	3.64
E39 (191–199)	EIWTHSTKV	1.33
E40 (247–255)	SLALMLLWL	1.18
E41 (245–253)	LLSLALMLL	1.20
No peptide		1.00

* MCF, mean channel fluorescence of FACS analysis with anti-HLA-2 mAb BB7.2. No peptide was utilized as baseline expression of HLA-A2 on T2. Results are expressed as a ratio of the MCF with the specific peptide as compared to no peptide.

IL-2. Standard cytotoxicity assays were performed with the TAL populations within a week of culture initiation to limit in vitro artifact. Figure 1 shows the results of OvTAL1 and OvTAL4 at multiple E : T ratios against all five of the FBP-derived peptides and unloaded T2 as a control. E39-pulsed T2 resulted in the best cytotoxicity with both of these effector populations. The recognition of this peptide by fresh, unmanipulated TAL suggests that these effectors have been previously exposed or primed to this epitope in vivo. To confirm these findings, all four TAL populations were tested against all five peptides in replicated assays performed in triplicate. These results are presented in Figure 2. Several patterns of recognition emerged from these assays, and are indicative of the different T cell repertoires present in the TAL populations. E37, a low-affinity binder, and E38, a high-affinity binder, were not significantly recognized in these assays, and, therefore, served as negative peptide

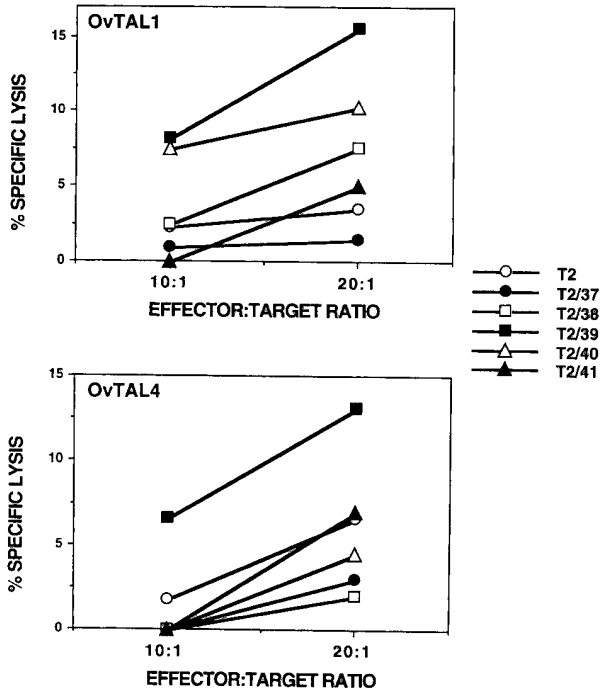

FIG. 1. Freshly cultured ovarian TAL recognize FBP peptides. Consecutive HLA-A2$^+$ ovarian TAL (OvTAL) were isolated from malignant ascites and cultured in IL-2 without specific stimulation. OvTAL1 and OvTAL4 were tested at multiple E : T ratios in standard 5-hour chromium-release assays for recognition of the HLA-A2$^+$ antigen-processing mutant T2,[16] when loaded with five FBP peptides (E37–E41) or no peptide (T2) as a negative control.

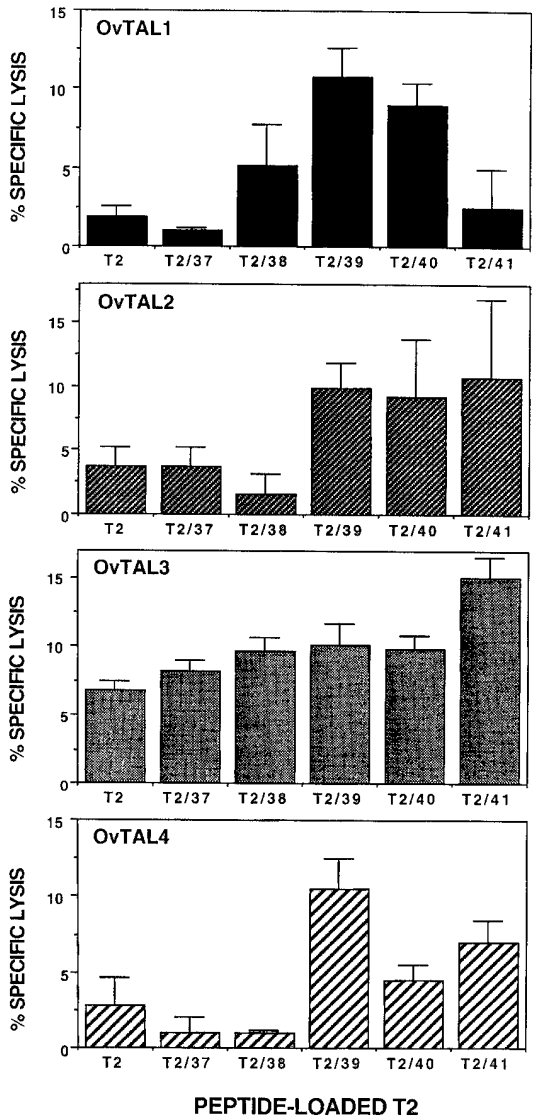

FIG. 2. FBP peptide recognition by consecutive ovarian TAL. Four consecutive HLA-A2$^+$ OvTAL populations were tested against T2 loaded with the five FBP peptides in standard 5-hour cytotoxicity assays. These assays were performed in triplicate at an E : T ratio of 20 : 1 and replicated for each effector. The results are expressed as % specific lysis ± SEM.

controls. E39-loaded T2 was the most consistently recognized target, being lysed by three of four cultures. The lysis data were significant overall when the data was pooled and compared to unloaded T2 or E37 and E38-pulsed T2 ($P < .05$). E39 appears to be the immunodominant FBP-derived peptide, whereas E40 and E41 may serve as subdominant peptides, because each reconstituted T-cell epitopes, with variable recognition.

The Specificity of TAL Recognition of the FBP-derived Peptide E39

To confirm the specific recognition of the HLA-A2/E39 peptide complex on T2 cells by CTL, inhibition assays were performed by initially adding the anti–HLA-A2 mAb BB7.2 to standard cytotoxicity assays. Figure 3*A* demonstrates the successful inhibition of OvTAL2 lysis of T2/39 at multiple E : T ratios by blocking HLA-A2 in 5-hour assays. These data were confirmed with two TAL populations in 20-hour ^{51}Cr-release assays to enhance the sensitivity of the method, as presented in Figure 3*B*. In the latter set of experiments, anti-HLA class I mAb, W6/32, also was utilized to confirm HLA class I presentation of the peptide to CTL because the W6/32 mAb is a more effective blocker of cytotoxicity than is BB7.2. These assays were repeated, and similar results were obtained.

Peptide Concentration-dependent TAL Recognition of FBP-derived Peptide, E39

To better understand the kinetics of CTL recognition of the E39 peptide, T2 cells were split into five parallel cultures and each pulsed with a different concentration of the peptide from 2 to 100 μg/mL for 1.5 hours before standard cytotoxicity assays with the same effector (OvTAL1) at a constant E : T ratio (Fig. 4). The optimal concentration with this low-affinity binding peptide was found to be 50 μg/mL, and half-maximal lysis occurred at the 2 to 5 μg/mL range. This is consistent with the range observed with HER2/neu peptides and most melanoma antigen peptides.[13,18,33] Cytotoxicity dropped considerably at higher concentrations, which has been a consistent finding in other peptide studies.

FBP-derived Peptides Are Naturally Processed Antigens

Cold target inhibition assays were performed to determine whether FBP-derived peptides reconstitute T-cell epitopes that are naturally processed and presented on ovarian tumor cells. Cold T2 were pulsed with E39 and then used to block the cytotoxicity of TAL populations for the ovarian cancer cell line SKOv3.A2. This cell line has been transfected with HLA-A2 in our laboratory and described previously.[31] Figure 5 shows that in multiple

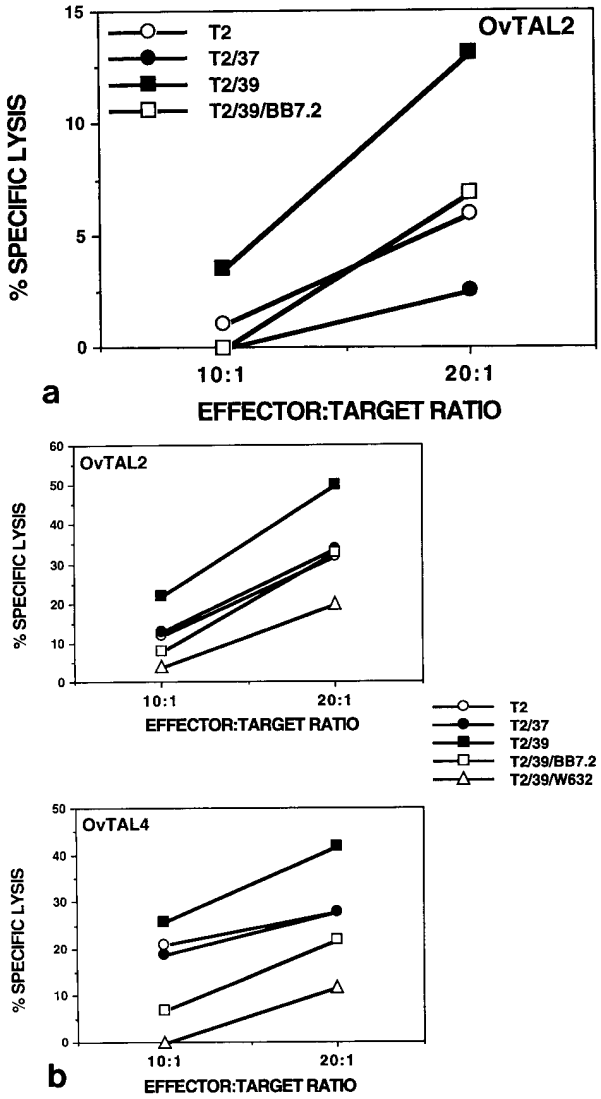

FIG. 3. OvTAL recognition of FBP peptide-loaded T2 is HLA-A2 restricted. mAb blocking assays were performed with the anti–HLA-A2 mAb BB7.2, and the anti-HLA class I mAb W6/32. (**A**) OvTAL2 was tested against T2 loaded with E39, E37, or no peptide in standard 5-hour cytotoxicity assays at multiple E : T ratios. The specific lysis of T2/39 was inhibited by adding BB7.2 to the wells 30 minutes before the assays. (**B**) Both BB7.2 and W6/32 were tested for inhibition of the cytotoxicity of OvTAL2 and OvTAL4 for T2/39 in 20-hour assays at multiple E : T ratios. T2 loaded with no peptide or E37 were used as negative controls. Results are expressed as % specific lysis. Results were confirmed in multiple assays.

assays, T2/39 effectively inhibited 20% to 40% of the tumor lysis by OvTAL1 and OvTAL4 ($P < .05$). These findings suggest that the CTL specific for this epitope contribute significantly to the recognition of this ovarian cancer cell line. Furthermore, these data demonstrate that FBP-derived peptides are naturally processed and presented antigens on intact ovarian tumor cells.

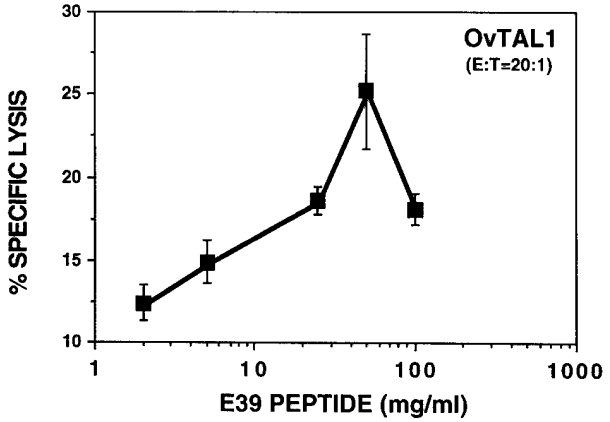

FIG. 4. OvTAL recognition of FBP peptide E39 is concentration dependent. OvTAL1 was tested against T2 incubated with increasing concentrations of peptide in standard 5-hour cytotoxicity assays at an E : T ratio of 20 : 1. The results are expressed as % specific lysis ± SEM.

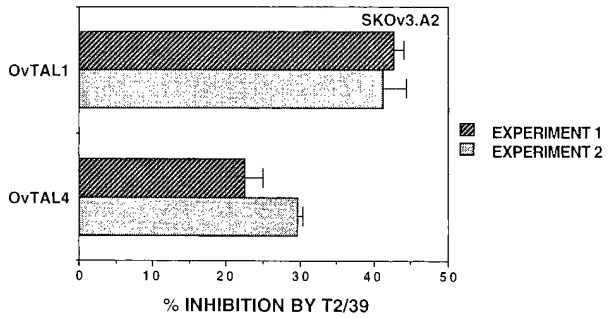

FIG. 5. The FBP peptide E39 is a naturally processed and presented antigen in ovarian cancer. Cold target inhibition assays were performed with OvTAL1 and OvTAL4 in replicated experiments. Cold T2 loaded with E37 (negative control peptide), E39, or no peptide (T2) were tested at a cold : hot ratio of 15 : 1 for inhibition of the recognition of the ovarian cancer cell line SKOv3.A2[27] by OvTAL at E : T ratios of 20 : 1 in 20-hour chromium-release assays. Results are expressed as % inhibition by T2/39 compared to T2/37 and T2.

DISCUSSION

This study demonstrates that FBP is a source of antigenic peptides that induce an endogenous immune response, as shown by the ability of freshly isolated and unmanipulated TAL to recognize several of these peptides, particularly E41 and, most consistently, E39. These peptides were recognized in a HLA-restricted fashion, and the cytotoxicity was concentration dependent. Furthermore, E39 was shown indirectly to be a naturally processed and expressed antigen on the ovarian cancer cell line SKOv3.A2, because peptide-loaded T2 could significantly inhibit CTL killing of this cancer line. Together these data strongly suggest that FBP is an endogenous TAA and is the source of antigenic peptides recognized by TAL in ovarian cancer.

Ovarian cancer has served as an extremely important model for the study of the immune response to epithelial cancer, as demonstrated by the work of multiple groups.[5–9] Most importantly, the findings have been extended to other less studied and technically more challenging epithelial tumor models. Furthermore, many findings in melanoma have been confirmed for epithelial cancers using this model. For example, we now know that an endogenous cellular immune response does exist in a variety of epithelial cancers, and that this response involves the specific recognition of antigenic peptides presented by HLA molecules, specifically HLA-A2, to specific TCR on tumor-infiltrating or tumor-associated CTL.[7,9,11] Unlike melanoma, investigation into the identity of these peptides has so far resulted in only one confirmed TAA, as defined by recognition by cellular immunity. Only the protein product of the oncogene HER2/neu has been shown to serve as a source of endogenously recognized antigenic peptides.[13,18] Unfortunately, this protein is overexpressed in only 30% of all ovarian and breast cancers.[21] The advancement in anticancer vaccine research has been swift in melanoma and has been fueled by the ready supply of multiple commonly expressed TAA.[33] For further development of potentially widely applicable epithelial cancer vaccines, more CTL-recognized TAA must be found for epithelial tumors.

FBP originally was discovered from three independent lines of investigation. The LK26 antigen was identified with a mAb raised against the choriocarcinoma cell line Lu-75(c) by Rettig et al.[34] This antigen initially was found to be expressed in normal as well as malignant trophoblastic cells and eventually in ovarian carcinomas.[22] The MOv18 and MOv19 mAbs were raised against an ovarian carcinoma cell membrane preparation and initially were found to react with a cell surface glycoprotein with a molecular weight of 38 kd.[23] This protein was cloned and sequenced and found to be a high-affinity FBP.[35] Likewise, the latter protein also was characterized from placenta and KB carcinoma cell lines.[36] The LK26 antigen eventually was found to be closely related or identical to the MOv18/MOv19 antigen.[22]

The distribution of FBP expression is extremely interesting and is relevant for immunotherapy. This protein is expressed in some normal specialized epithelium, such as choroid plexus, lung, thyroid, kidney, and sweat glands, but at very low levels.[25] The highest levels of expression of FBP have been found in ovarian carcinomas, and in several independent studies more than 90% of all ovarian carcinomas tested expressed elevated levels of this protein.[22,24] The levels of overexpression have

been shown to be more than 20 times that of normal tissue,[25] and in one study were reported to be as high as 80 to 90 times that of normal tissue.[24] In addition, multiple tumor types have been shown to overexpress the LK26/FBP antigen, including 10 of 11 endometrial cancers, six of 27 colorectal cancers, 11 of 53 breast cancers, six of 18 lung cancers, nine of 18 renal cell cancers, three of three lung carcinoids, and four of four brain metastases from breast cancer. Mesotheliomas, lymphomas, sarcomas, and neuroectodermal tumors were either negative or rarely positive for FBP expression.[22]

The fact that this TAA is so widely and differentially expressed among multiple epithelial tumor types makes it an ideal target for immunotherapy. Multiple attempts have been made to target therapeutic strategies toward FBP, including folate conjugates and antifolates.[28–30] Several studies have involved immunoconjugates or bispecific mAbs, similar to the studies targeting CA-125.[26,27] The use of mAbs against this and other similar cell surface antigens has been limited, because many of these mAbs have been raised against membrane extracts and may have less efficient recognition against the endogenously expressed conformational protein. Also, FBP is shed, and much of the available mAb is bound to circulating antigen. Finally, much of the tumor often is inaccessible to circulating mAbs, and they are rapidly cleared by the host. For these and other reasons, developing mAb-mediated therapies targeting FBP has been a challenge. However, the fact remains that most ovarian carcinomas drastically overexpress FBP, and this antigen is endogenously processed and presented for recognition by cellular immunity.

The FBP-derived peptides shown in this study to be recognized by ovarian cancer-associated CTL may be used in several different immunotherapeutic strategies. First, these peptides could be used to stimulate FBP-specific CTL in vitro for cellular therapy. Adoptive immunotherapy has been shown to reduce tumor burden significantly in up to 30% of end-stage melanoma and renal cell carcinoma patients with TIL.[37,38] In ovarian cancer, TIL in combination with chemotherapy had a synergistic effect, with better results than chemotherapy alone.[39] These results were obtained with uneducated and largely nonspecific TIL, and could be appreciably improved with highly specific CTL directed toward a known TAA. CTL induction studies with FBP peptides currently are underway in our laboratory.

These peptides also may form the basis of a peptide vaccine. Delivery systems for peptide antigens, including dendritic cells and viral vectors, currently are being investigated in melanoma with several ongoing studies.[40] Encouraging results have been reported with the efficient induction of cellular responses in vivo to melanoma-derived peptide antigens effectively delivered.[41,42] We, too, have been investigating HER2/neu-derived peptides in similar strategies and have found efficient CTL induction in vitro with peptide-pulsed dendritic cells.[43] We currently are studying the E75 peptide in clinical trials and virally delivered peptide in vitro. Similar studies will soon be initiated with FBP-derived peptides.

The successful development of a vaccine against epithelial cancer rests on the identification of widely expressed, CTL-recognized antigens that are either exclusively or highly associated with cancerous cells. FBP appears to be the second such known antigen and may be superior to HER2/neu, given its distribution and level of expression as a target for cellular immunity.

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Ovarian and breast cytotoxic T lymphocytes can recognize peptides from the amino enhancer of split protein of the *Notch* complex

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Abstract

In this study we investigated recognition by ovarian tumor associated lymphocyte (OVTAL), and breast tumor associated lymphocytes (BRTAL), of peptides corresponding to the sequence 125–135 of the Aminoenhancer of split (AES) protein. Three of these peptides designated as G75:AES1/2 (128–135), G60: AES1/2 (127–137) and G61: AES1/2 (125–133) correspond to the wild-type AES sequence, while the fourth G76:GPLTPLPV, AES1/2 (128–135) corresponds to a variant sequence of the peptide G75 with the N-terminal Leu substituted to glycine. These sequences were chosen for study because mass-spectrometric analysis (MS) of a CTL active HPLC peptide fraction eluted from immunoaffinity precipitated HLA-A2 molecule, revealed: (a) the presence of an ion with a mass-to-charge ratio (m/z) of 793 which was more abundant than other ions of similar masses; (b) the tentatively reconstituted sequence of the ion 793 matched the sequence of peptide G76. We found that AES peptides G75 (128–135) and G76 (128–135) (L128G) reconstituted CTL recognition at concentrations ranging between 200–500 nM. These concentrations are lower than concentrations reported to activate effector function of CTL recognizing other epithelial tumor Ag. Furthermore, analysis with cloned $CD8^+$ T cells indicated that G75 and G76 were not cross-reactive specificities, suggesting a key role for the N-terminal residues of the variant peptide in dictating specificities. Since the AES proteins are part of a set of transcriptional repressors encoded by the Enhancer of split [E(spl)] genes, and since these repressors are activated to suppress cell differentiation in response to *Notch* receptors signalling, the AES peptides may represent a novel class of self-antigens that deserve further consideration as tumor Ag in epithelial cancers.

Keywords: Notch; AES; CTL; Epitopes; Breast; Ovary

1. Introduction

Advances in diagnostic and conventional therapies have led to earlier detection and improved quality of life for cancer patients. However, the establishment of drug resistance has raised the need for novel approaches to therapy of tumors. During recent years, studies on human cancer antigens (Ag) have identified peptides from self-proteins that are recognized by cytotoxic T lymphocytes (CTL). Most of these antigens have been discovered in the melanoma system (Houghton, 1994, Boon van der Bruggen, 1996). The expression of these CTL epitopes has been found to be dependent in some instances but not in others on the levels of MHC-class I expression (Rivoltini et al., 1995; Fisk et al., 1997). Regardless of the presence of CTL in tumor infiltrations, the disease progresses suggesting that this CTL response is too weak to mediate tumor regression. Furthermore, tumor progression may be dependent on an immunoselection process, characterized by the fact that tumor cells that lack expression of certain antigens may gain a proliferative advantage (Seung et al., 1995; Kono et al., 1997). Thus, the elimination of tumor cells expressing

Abbreviations: OVTAL, ovarian tumor associated lymphocytes; BRTAL, breast tumor associated lymphocytes; CID, collision-induced dissociation; AES, Amino enhancer of split; TLE, Transducin-like enhancer of split; PCR, Polymerase chain reaction; m/z, mass-to-charge ratio; U, mass unit; MS, mass spectrometry; w.t., wild-type; MCF, mean channel fluorescence.

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defined epitopes should allow unaffected growth of other tumor cells that do not express these epitopes.

Ag specific cancer vaccines may provide a complementary approach to traditional therapies if efficient targeting of cytotoxic effectors can be accomplished. In contrast with melanoma (Kawakami et al., 1994a,b; Castelli et al., 1995; Cox et al., 1994), there is little information on the nature of tumor Ag present on epithelial tumors such as breast and ovary which affect a large segment of the population. To this moment, the spectrum of tumor Ag and corresponding CTL epitopes in breast and ovarian cancer is limited. It includes mainly the deglycosylated Muc-1 core peptide epitope (Jerome et al., 1993, Ioannides et al., 1993a), and HER-2 epitopes, the latter detectable, in general, in tumors with HER-2 overexpression (Ioannides et al., 1993b; Fisk et al., 1995; Peoples et al., 1995; Yoshino et al., 1995).

Characterization of additional tumor epitopes are needed since it may allow development of polyspecific cancer vaccines, which can target a larger population of antigenically distinct tumor cells. Identification of such epitopes on epithelial tumors appear to encounter difficulties. Muc-1 and HER-2 were initially targeted for study because of their different post-translational modification (Muc-1) or overexpression (HER-2) on tumor cells compared with normal tissues (Ioannides et al., 1995; Peoples et al., 1995). There is little information on other tumor genes and proteins expressed on cancer cells that can provide the focus of study of CTL recognition using synthetic peptide mapping.

An additional difficulty in characterization of novel cancer antigens rests in the limitations in the availability of primary tumor in the large amounts ($>10^{10}$ cells) needed for biochemical characterization of extracted peptides (Cox et al., 1994; Slingluff et al., 1993; den Haan et al., 1985; Udaka et al., 1992). This leaves, at this time, as the only feasible approach for novel tumor Ag identification, the use of tumor lines as primary source of tumor peptides. Mapping of active peptide fractions from acid treated tumors using breast and ovarian CTL isolated from tumor infiltrating lymphocytes (TIL) may allow focusing the search on candidate CTL epitopes. The fact that these CTL are not induced or expanded by stimulation with the tumor line should allow identification of pre-existing epitope specificities in the patient. These peptides can then be sequenced by mass-spectrometry (MS) and the candidate sequences derived from integration of resulting daughter ions tested as synthetic peptide equivalents to induce activation of CTL effector functions.

We have recently used this approach for characterization of the common peaks of naturally processed peptides shared between an ovarian tumor line (SKOV3.A2) and a freshly isolated ovarian tumor. We found in addition to a number of overlapping peaks of biological activity, several non-overlapping peaks of activity (Fisk et al., 1997a,b). The presence of the overlapping peaks is of interest because it suggests that such epitopes may have been presented on the original primary tumor and were stimulators for CTL.

MS analysis of the ions present in the peak *B*2 of overlapping activity corresponding to the HER-2 peptide E75 (Fisk et al., 1997a,b) revealed the presence of a number of ions (Fisk et al., 1997b). The signal intensity of several ions in a number of fractions matched the pattern of CTL activity of two ovarian tumor associated CTL-TAL lines (Fisk et al., 1997b). One of these ions of m/z: 792.9 (and further designated as ion 793) was selected for sequencing by MS because its signal intensity was significantly higher than that of the other ions of similar or higher masses suggesting an abundant peptide. Reconstitution of the 793 sequence suggested several possible peptides, of which, the best match 7/8, was found within the sequence responsive amino acids 128–135 of the amino enhancer of split protein (AES-1/2) (Miyasaka et al., 1993) of the *Notch* complex (Stifani et al., 1992) associated with cell differentiation (Artavanis-Tsakonas et al., 1995). Synthetic peptides of these sequences were found to reconstitute recognition of two ovarian and two breast TAL lines, isolated from ascites or pleural effusions respectively, suggesting that they may provide an additional target for tumor specific CTL.

2. Materials and methods

2.1. Cells and cell lines

The ovarian tumor line SKOV3.A2 has been previously described (Fisk et al., 1995). Other targets used in these studies consisted of freshly isolated breast and ovarian tumors from malignant effusions. BRTAL and OVTAL ascites or pleural effusions occurring in patients with advanced breast or ovarian carcinomas were isolated from ovarian ascites (OVA-TAL) or breast pleural effussions and ascites (BRTAL). Isolation of tumors, lymphocytes and lymphocyte culture was performed as previously described (Fisk et al., 1995). CTL assays to determine recognition of peptide pulsed T2 cells, tumor lysis and cold-target inhibition assays followed the previously reported procedures (Fisk et al., 1994, 1995). Tumor peptide extraction, HPLC fractionation using two acetonitrile gradients and CTL epitope reconstitution assays have been described (Fisk et al., 1997a,b). Effectors were generated by culture of OVTAL and BRTAL in RPMI media containing 10% FCS and 50 U/ml (Cetus) of IL-2 (complete RPMI medium).

For separation of $CD8^+$ cells, freshly isolated OVTAL and BRTAL were propagated in RPMI 1640 medium containing 10% FCS, antibiotics and 50 U/ml of IL-2 (Cetus) for one week. Afterwards the $CD8^+$ cells were isolated using magnetic beads (Dynabeads, Dynal, Oslo,

Norway) and cultured with the same conditions. For the purpose of limited cloning, the $CD8^+$ cells were plated in 96-well plates using binary dilutions ranging from 20–5000 cells/well in the presence of irradiated PBMC from $HLA\text{-}A2^+$ donors and alternatively stimulated with OKT3 mAb and PHA. None of these cultures was stimulated with peptides or tumor cells. Furthermore, tumor cells were not used as feeders. Two to three weeks later, the wells were scored for growth. Using this procedure, we found that in most instances proliferating cultures of $CD8^+$ cells resulted from wells initially seeded with 80–160 cells/well but not from wells where the $CD8^+$ cells were seeded at lower densities (Ioannides et al., 1991). Thus we assumed that from wells containing 80 cells or more, at least one $CD8^+$ cell was able to proliferate, while such a $CD8^+$ cell was absent from wells containing half the cell number (i.e. 40 cells) because proliferation was not observed from cultures started with 40 $CD8^+$ cells/well. Of the 'clonal' cultures by the above approach, the ones that maintained stable growth for at least one month from the initiation of the limited cloning procedure were tested for peptide recognition. Since these cultures were not recloned they are designated as T cell lines.

2.2. Mass-spectrometry

Five consecutive HPLC fractions (fractions 38–42) corresponding to the peak B2 of activity of peptides eluted from the immunoaffinity separated HLA-A2 molecules from SKOV3.A2 cells were analysed by MS for the presence of ions, whose relative abundance matched the CTL activity of two ovarian CTL-TAL lines (Fisk et al., 1997b). The single-charged ion of $m/z = 793$ was found in fractions 40 and 41, but not in the other fractions. Identification of the ion composition of the peak B2 fractions has been recently reported (Fisk et al., 1997b). Detailed methodological approaches to ion analysis and MS sequencing have been reported (Fisk et al., 1997b). Sequencing of the ion 793 was performed by the Analytical Biochemistry Center of the University of Texas Medical School in Houston, Texas. Collision-induced dissociation (CID) mass-spectra were obtained with a Finnigan MAT TSQ70-triple-quadrupole instrument upgraded with TSQ700 software and a 20 kV conversion dynode electron multiplier. For ion scans, the resolution of the first quadrupole (Q.1) was adjusted to allow transmission of +2 U from the center of the mass of interest. A peak width of 1 U was used for post-acquisition spectral averaging and quantitation by manual integration of selected chromatograms.

Sequence reconstitution was also performed using the computer program PEPSEQ version 1.2 (Sampson et al., 1995). Although this program identifies a large number of potential candidate sequences, it also focused the search for the candidate sequence by allowing increasing stringency. Peptide sequences were identified based on the concordance of determined and predicted mass-values for peaks of ions in a candidate sequence. The concordance was determined based on the lowest deviation between experimental and theoretical values for the respective ions and defined as the lowest score/peak ratio (Fisk et al., 1997b).

2.3. Synthetic peptides

Synthetic peptides of candidate ions prepared were: G76 (AES-1: 128–135,GPLTPLPV), G75 (AES-1: 128–135, LPLTPLPV). The peptide of the same sequence with G76 with the last two C-terminal residues inverted was designated G57:GPLTPLVP. To examine the possibility that the epitope formed is part of a longer peptide, the following peptides were prepared by extending the sequence AES-1,128–135, by two residues at C-terminus, i.e. G58:GPLTPLPVGL, G59:PLTPLVPGL, G77:GPLTPLPVGL and G78: PLTPLPVGL. To examine the possibility that the N-terminal extended sequence forms CTL epitopes two peptides were prepared G60: (AES-1, 127–135): ALPLTPLPV, and G61:(AES-1, 125–133): ALALPLTPL. The numbers assigned for the position of these peptides in the sequence follow the sequence of AES-1 protein. The sequence in the AES-2 protein is identical in this area, but the position of the sequence is N-terminally shifted by 11 residues in AES-2 compared with AES-1(21). AES-1 and AES-2 proteins, resulted likely from alternative splicing of the same precursor mRNA (Miyasaka et al., 1993; Mallo et al., 1995). For clarity, only the AES-1 sequence is referred to in this study. These peptides were prepared by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center and purified by HPLC to >95% purity. The codes used to identify peptide in this study were assigned by the same laboratory. Peptides G76, G75, and G57 were sequenced by CID as described above, and their fragment ions spectra were compared with the ions resulted from the natural ion 793. To facilitate presentation, the amino acids substituted from the natural sequence or groups reverted from the natural sequence are underlined. All other peptides used in these studies have been previously described (Fisk et al., 1995).

2.4. Immunofluorescence

HLA-A2 stabilization assays were performed using the T2 line as indicator as we previously described (Fisk et al., 1996). Expression of HLA-A2 was determined using the HLA-A2 specific mAb, BB7.2 and MA2.1. Hybridomas secreting these mAb were obtained from ATCC. The stabilizing ability of AES peptides indicative of their HLA-A2 affinity was determined from their ability to enhance HLA-A2 expression after overnight culture with T2 cells as described (Fisk et al., 1996).

3. Results

3.1. Characterization of the sequence of the ion 793

The ion 793 was sequenced by mass-spectrometry using collision induced dissociation (CID). To obtain a candidate peptide sequence, the resulting daughter ions were first examined using the program PEPSEQ version 1.2. To focus the search for a sequence, the stringency was increased after each round of analysis. At a tolerance of 0.5 U (<0.1% deviation from the mass of ion 793) only six candidate sequences were selected by the PEPSEQ program. A search in nucleic acid and protein data bases using candidate peptides containing in the sequence either Leu (L) or Ile (I) or both revealed that of the six sequences only the sequenceGPLTPLPV gave the highest number of matches (7/8 matches) with a known sequence LPLTPLPV. This sequence corresponded to amino acids 128–135 of the amino enhancer of split (AES) protein. The gene for this protein is a member of the *TLE* (transducin-like enhancer of split) genes (TLE complex) associated with the *Notch* complex (Miyasaka et al., 1993). The human AES and TLE-1,-2, and -3 proteins show significant sequence homology in other areas but differ in this particular area (e.g. human TLE-1:139–150 sequence is GPPVPLPHPSGL (Stifani et al., 1992).

To characterize the correspondence between the experimentally determined and predicted sequence for ion 793, synthetic peptides G76:GPLTPLPV. G57:GPLTPLVP, and G75:LPLTPLPV were prepared. These peptides were then sequenced by CID. The predicted mass values for the *y* and *b* ions of peptide G76 are listed below (Table 1). These values were highly similar to the values predicted for peptide G57 ions, in which the last two C-terminal amino acids are reverted from the sequence of G76 (data not shown). Sequencing of peptide G76 showed a good correlation between the experimentally obtained and predicted mass values for 5/8 *b*, 4/8 *y*, 2/8 *bo*, 4/8 *yo*, and 2/8 *a*, G76 daughter ions (Fig. 1A). Because the amount of HPLC sample used for sequencing was limited, to integrate the sequence the resulting daughter ion masses of the peak 793 were compared with respect the position and signal intensity with the predicted masses of the G76 daughter ions *b* and *y* and the corresponding *a*, *bo*, and *yo* ions (Papayannopoulos, 1995). The experimentally obtained sequence data for peptide G76 and ion 793 are shown side by side in Fig. 1A. Although some major daughter ions species of peptide G76 were not detectable in the 793 spectrum, seven of eight *b* ions and four of eight *y* ions of 793 were present and found to match within 1 U, with the predicted values of the corresponding ions for peptide G76:GPLTPLPV. Furthermore, peaks corresponding to 4/8 *yo* ions, 3/8 *bo* ions and 2/8 *a* ions of the peptide G76 were also present in the spectrum of fragment ions of 793 (Fig. 1A).

To identify a candidate peptide sequence for the ion of *m/z* 793 we used in the interpretation of the data first the candidate *b* ions, then the candidate *y* ions. Expanded CID spectra for the ion of *m/z* 793 are shown in Fig. 2A and 2B. These spectra were obtained by normalizing the data to the next most abundant ions other than *m/z* 496 and 793 which were the most abundant (Fig. 1A). This allowed the presence and position of the smaller peaks to be determined more accurately.

From integration of the values for the candidate *b* ions *m/z* (58.2, 155.5, 369.3, 465.3, 577.0, 679.1 and 775.2) the candidate sequence appears as GPZZPLVP, (Z symbolizes an unknown residue). Then we examined the candidate *y* and *yo* ions. *b* and *bo*, *y* and *yo* ions differ in mass by a molecule of water (18 U). Thus from *y*/*yo* ions of *m/z* 793/775, –/717, 639/621, 526/508, 328/–, 216.9/200 (?) the first three N-terminal residues can read as GPL, while the last two C-terminal residues may read PV/VP (See also candidate ion $y2^{1+}$ *m/z* 216.9 and yo2 *m/z* 199–200. Based on the difference in mass between candidate *b*4 and *b*2 ions [369.3–155.5 = (213.8)] if Leu or Ile is present at P3, then the P4 residue may be Thr (Papayannopoulos, 1995). Thus integration of *y* ions led again to a candidate peptide of sequence either GPLTPLPV or GPLTPLVP (Papayannopoulos, 1995). Our data do not allow for unambigous assignment of the order of the last two amino acids. Therefore, the sequence of ion 793 deduced from *b* and *y* ions also shows a good match (7/8) with the sequence of AES-1/2 proteins in the area 128–135, initially tentatively identified using the PEPSEQ program. The signal for N-terminal Leu (mass 113) corresponding to the wild-type AES peptide was not detected in the spectrum of the ion 793 but was detectable in the spectrum of the synthetic peptide G75 suggesting that its

Table 1
Predicted masses of daughter ions of the peptideGPLTPLPV: AES-1 (128–135)*

No.	Seq.	*a*	*b*	*bo*	*d*	*y*	*yo*	No.
1	Gly	30.0	58.8	40.0	75.1	793.5	775.5	8
2	Pro	127.1	155.1	137.1	172.1	736.5	718.5	7
3	Leu	240.2	268.2	250.2	285.2	639.4	621.4	6
4	Thr	341.2	369.2	351.2	386.2	526.3	508.3	5
5	Pro	438.3	466.3	448.3	483.3	425.3	407.3	4
6	Leu	551.4	579.4	561.3	596.4	328.2	310.2	3
7	Pro	648.4	676.4	658.4	693.4	215.1	197.1	2
8	Val	747.5	775.5	757.5	792.5	118.1	100.1	1

* The values in the vertical columns indicate the residue masses of the peptides resulting from degradation of peptide G76, in the free amino and free acid form, starting from each terminus. The *bo*/*yo* ions differ from the corresponding *b* and *y* ions by the loss of one molecule of water (i.e. 58 – (16 + 2) = 40), while the *a* ions differ in mass from the *b* ions by the loss of a carbonyl group i.e. 58 – (16 + 12) = 30). The exact mass of peptide G76 (C38 H64 N8 O10) is 792.47. The exact mass of peptide G57 with the C terminal Pro-Val group reverted as compared to G76 is the same with G76.

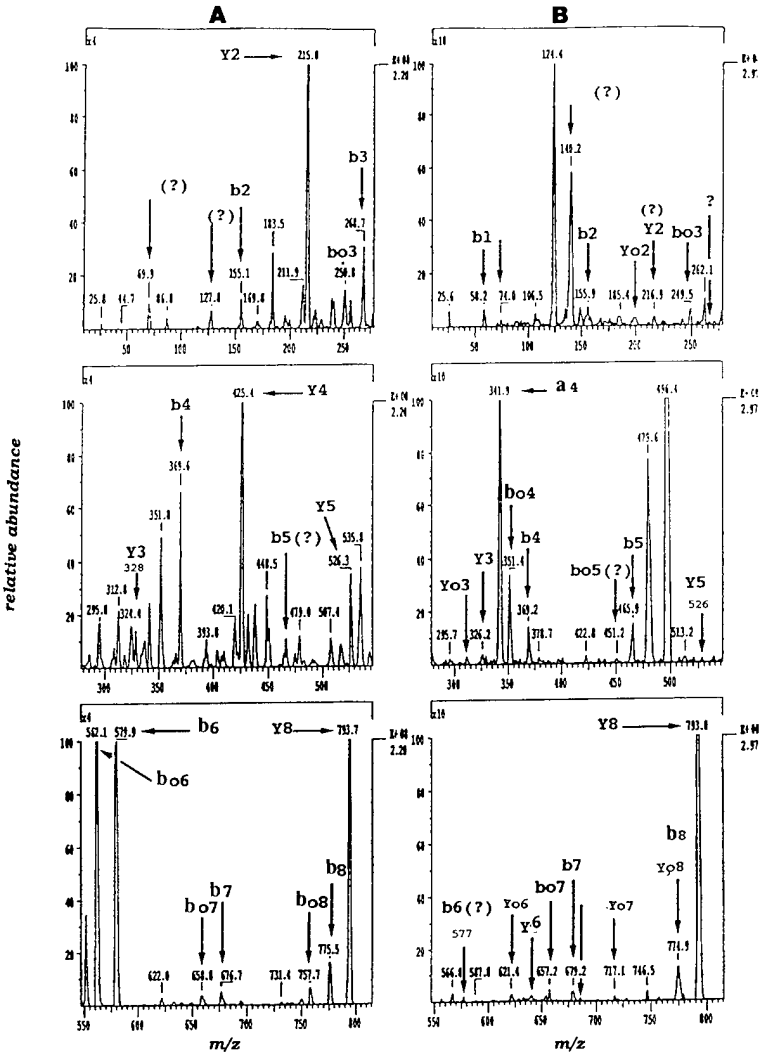

Fig. 1. CID spectra of the peptide G76 **(A)** and of the ion 793 **(B)**. The ions with similar positions are marked with arrows. The tentative assignment of the ions was made by comparing the experimental values with their predicted values listed in the Table 1. The ions which differ from the predicted values by more than 1.5–2 U are indicated with the mark(?). Note the absence of *b*1 and *y*1 from the spectrum of peptide G76 and the presence of the strong ionizing ion 496.4 in the spectrum of the ion 793. The experimental conditions were as described in the Materials and methods.

lack of detection does not represent a limitation of the sequencing method (data not shown).

The CID spectrum of the ion 793 also shows the presence of strong signals from ions *m/z* 124, 140, 496, as well as of signals from other ions at weaker intensity. The first three ions 124, 140, 496.4 may represent impurities (likely phospholipids, unpublished observations). This is because MS/MS spectra from other ions *m/z*: 453, 609, 638, 787, 1008 and 1017 detected in this peak (Fisk et al., 1997) showed a similar pattern of background interference. For example the ion 140 was found in the CID spectra of ions of *m/z* (453, 609, 638, 787, 1008, and 1017), the ion 124 was present in the CID spectra of ions *m/z* 609 and 638, while the ion 496 was present in the CID spectra of the ions of *m/z* 609, 638, and 786 (data not shown).

It should be noted that these peptides were isolated as mixtures from very complex biological matrices and were not expected to be pure. Ions 124 and 140 did not correspond to dipeptides. Ion 496 was the ion with the strongest intensity in the spectrum, stronger than that of any *b* and *y* ion and even stronger than that of ion 793. Some of the other ions present may indicate the presence of an additional peptidic component at lower density, e.g. ions 279/261, 479, 566/538, and 653. Thus, at this time we cannot rule out the possibility that the active fractions contain a mixture of active and inactive components of peptide nature not all of which correspond to AES sequences.

Clarification of the presence of the missing ions in 793, and certification of its peptidic nature would have required additional material which would in turn have meant growing more tumor cells on an even larger scale ($>40\times10^{10}$ cells). However, the important question was

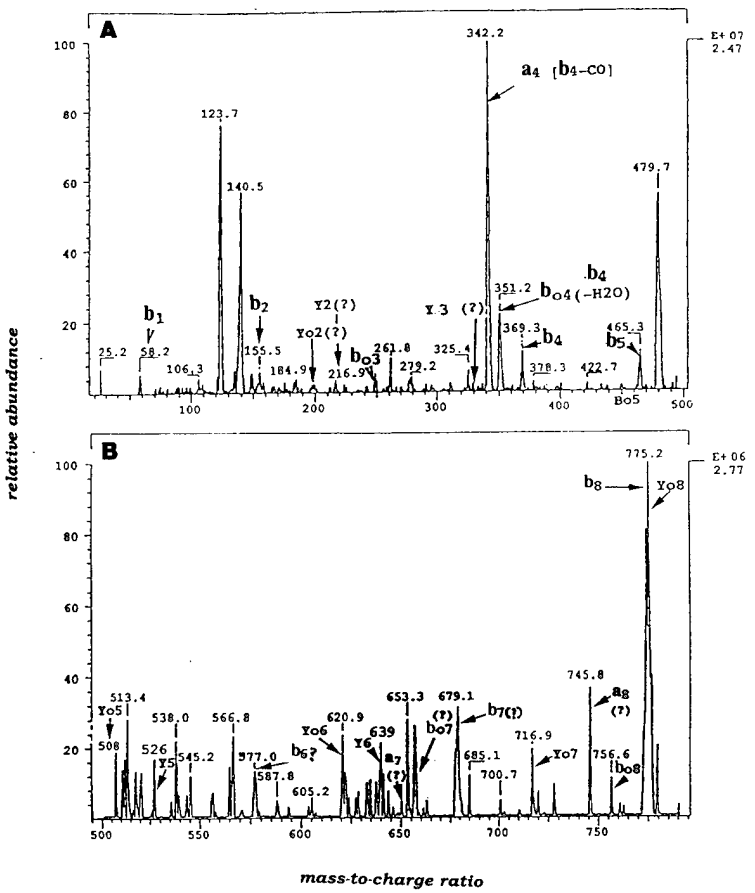

Fig. 2. Enlarged presentations of the CID spectra of the ion 793, after leaving out **(A)** the ions of m/z 496.4 and **(B)** the ion of m/z 793.0. Arrows indicate the positions and designation of the candidate Y and B ions by comparison with the values in Table 1. Note the low signal for the ions in the expected positions for $b1$, $b2$, $b3$, and the absence of $y1$, $y3$, $y4$ and $y7$.

whether a peptide with this sequence is functional, and the significance of its function. We hypothesized that if the proposed sequence for the ion 793 corresponds to a peptide, and this peptide is functional then it should be able to: (a) bind to HLA-A2 and (b) activate effector pathways by $CD8^+$ T cells. Therefore to establish the significance of the ion 793 and its related peptides, we decided to investigate recognition by breast and ovarian CTL of AES peptides encompassing the amino acids 125–135.

The area AES-1, 125–135 includes two overlapping sequences ALPLTPLPV (127–135) and ALALPLTPL (125–133) that contain HLA-A2 binding motifs and strong P1P2 anchors. Since the proposed sequence for the ion 793 corresponded to an N-terminally mutated AES peptide of the sequence 128–135, we prepared in addition to G76(GPLTPLPV) and G75(LPLTPLPV), the peptides G60(AES-1 (127–135 ALPLTPLPV) which differs from G75 by the presence of N-terminal Ala, and the peptide G61 (AES-1 (125–133) which overlaps in part (LPLTPL) with the other three peptides. It should be noted that the m/z of AES-1 peptides, 128–135 (designated as G75), 127–135 (G60), and 125–133 (G61) are different and higher than 793, e.g. m/z of G75 = 849.1. HPLC analysis indicated that they elute with distinct retention times (data not shown). Thus it is unlikely that they are present in the same HPLC peak.

3.2. Stabilization assays

To address the question of the ability of these AES peptides to bind HLA-A2 we performed T2 stabilization assays. The results in Table 2 show that, with respect reactivity with BB7.2 mAb, the epitopes formed by peptides G76 and G75 are likely to be conformationally different (Fisk et al., 1996). Staining with MA2.1 mAb revealed a higher stabilizing ability of HLA-A2 by G76 than did staining with BB7.2 mAb. In contrast, the levels of HLA-A2 stabilization detected by MA2.1 and BB7.2 mAb were similar for the peptide G75 corresponding to the wild-type AES-1 (128–135) sequence. The possibility that G75 and G76 are conformationally different epitopes was strengthened by the fact the peptides G77 and G78 (AES-1, 128–137) which differ from G76 and G75 by the presence of the C-terminal group Gly-Leu, induced a similar pattern of staining with G76 and G75 by MA2.1

Table 2
Stabilization of HLA-A2 expression on T2 cells by AES peptides

Code	Sequence	MCF-R[a] MA2.1	MCF-R[a] BB7.2
G76:	G P L T P L P V	1.76	1.03
G75:	L P L T P L P V	1.53	1.52
G77:	G P L T P L P V G L	1.51	0.96
G78:	L P L T P L P V G L	1.44	1.2
G60:	A L P L T P L P V	4.66	N.D.
G61:	A L A L P L T P L	3.06	2.80
G57:	G P L T P L V P	1.29	N.D.
G58:	G P L T P L V P G L	1.07	N.D.
G59:	P L T P L V P G L	1.58	N.D.

[a] represents the mean channel fluorescence ratio (MCF-R), between the MCF corresponding to T2 cells incubated with any of the peptides and T2 (control) that have not been incubated with peptide (T2/NP). Each peptide was used at a concentration of 20 μg/ml.

and BB7.2 mAb. C-terminal extension of the epitope (as in peptides G77 and G78) did not increase the binding ability of the peptide to HLA-A2. The stabilizing ability of peptides G57 and G58 with the C-terminal group reverted to VP (P7–8) was lower than that of the corresponding w.t. peptides G76 and G77.

Peptides G60 (127–135) and G61 (125–133) showed significantly higher binding affinity for HLA-A2, than did all other peptides tested. The stabilizing ability of G60 was comparable with the stabilizing activity of the high-affinity HLA-A2- associated CTL epitope influenza-matrix (M:58-66) peptide. These results suggested that peptides containing the group GP at P1-2 can bind and stabilize HLA-A2 with low affinity even though they do not express the canonical HLA-A2 anchors. This also suggested that one candidate natural peptide(s) of the G76 sequence can be presented by HLA-A2.

3.3. Recognition of AES peptides by ovarian and breast tumor reactive CTL

AES sequence analysis indicate that in the same area (125–135), based on HLA-A2 binding motifs, several overlapping candidate CTL epitopes (G75, G60, G61) may be present. The L → G (128) change in the AES may reflect a variant member of the AES family as reported for other Ag (den Haan et al., 1995). This raised the question whether the w.t. peptide G75 and/or the variant peptide G76 are recognized by TAL. We decided to investigate in parallel the recognition of a w.t. candidate CTL epitope (represented by peptide G75), of the natural candidate CTL epitope (G76), and two overlapping nonamers containing canonical HLA-A2 anchors corresponding to the w.t. AES sequences 127–135 (G60) and 125–133 (G61).

Since ion 793 was found to be expressed by an ovarian tumor line, we first tested recognition of AES peptides by ovarian TAL. To determine whether any of these AES peptides are recognized by ovarian TAL (OVTAL) we first analysed the concentration-dependent recognition of G76 (w.t.), G76 (natural) and of the overlapping peptides G60 and G61. All these peptides shared the motif PLTPL. The effectors were generated by brief culture in medium containing IL-2 of OVTAL from HLA-A2$^+$ donors. These OVTAL were not restimulated with tumors during in vitro culture to avoid changes in Ag specificity. Both G75 and G76 were recognized at a concentration of 1 μg/ml by OVTAL-1 (Fig. 3A). Of interest, at 5 μg/ml G76 recognition indicated signs of saturation. Recognition of G60 and G61 was borderline and not significantly different from controls (data not shown). Since G75 and G76 differ only in the N-terminal residue we isolated CD8$^+$ lines from OVTAL-1, and tested recognition of G75, G76 as well as the lysability of SKOV3.A2 cells by these effectors. The results (Fig. 3B) show that the line OD8 recognized G75 but not G76. This line also recognized SKOV3.A2 cells suggesting that a similar epitope with the one formed by G75 may be present in the tumor cells. Another line, OF81, isolated from the same donor recognized G76 at concentration as low as 0.2 μg/ml but did not recognize G75 (Fig. 3C). The line OF81 also recognized SKOV3.A2 cells.

To evaluate whether OVTAL could recognize AES peptides G60 and G61, that bind HLA-A2 with high affinity, lysis of T2 cells preincubated with each of the low A2 affinity (G75, G76) and high affinity peptides (G60, G61) was tested in the same experiment over a range of concentrations by OVTAL from a second donor (OVTAL2). Both G76 and G75 were recognized by OVTAL-2 at lower peptide concentration (5 μg/ml) better than G60 and G61 (Fig. 4A–D). At 50 μg/ml recognition of G75 and G76 in fact decreased. G60 and G61 were recognized by OVTAL-2 although required significantly higher concentrations (25–50 μg/ml) than G75 and G76. Taking into consideration that G60 and G61 have significantly higher affinity for HLA-A2, than G75 and G76 this suggested that the affinity of TCR for the G76-HLA-A2 and G75-HLA-A2 epitope was higher than the affinity of TCR for the G60/G61- HLA-A2 epitopes. Since G75 and G60 differ only at their N-terminal Ala, this further indicates that the epitope(s) formed by G76/G75 were preferentially recognized over G60 and G61.

Similar results were observed when G75, G76, and G61 were tested for recognition by breast BR-TAL-1. G76 was better recognized than G75 and G61 (Fig. 5A–C). Therefore, the results, using two ovarian and one breast TAL lines, suggest that both octamers, the w.t. and the variant AES epitopes are recognized by TAL, with higher affinity than the overlapping nonamers G60 and G61. These results also suggest that epitope(s) recognized by the TCR of these TAL may be located closely to the N-

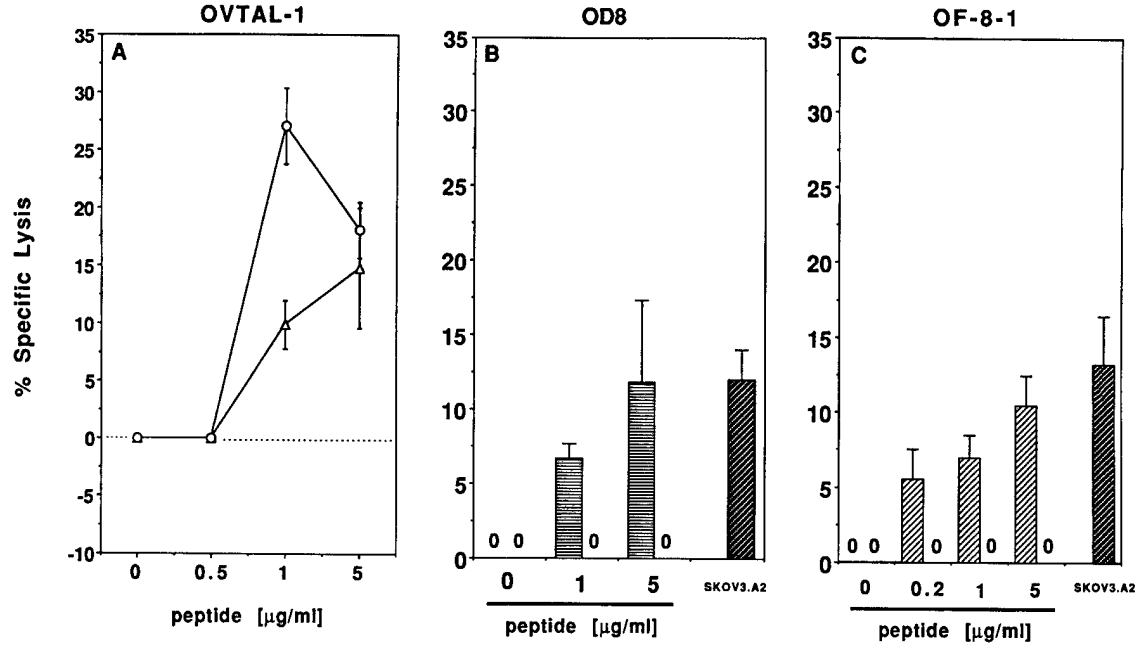

Fig. 3. **(A)** Concentration dependent recognition of AES-1 peptides G75 (0) and G76 (Δ) by OVTAL-1 at an E:T ratio of 10:1. Recognition of G75 (▤), G76 (▨) and of SKOV3.A2 (▨) by two $CD8^+$ lines, OD8 **(B)** and OF81 **(C)** derived from OVTAL-1. E:T was 8:1. Recognition by OVTAL-1, OD8 and OF81 was determined in separate experiments **(A)**. One of two experiments with similar results is shown. All experiments were performed in triplicate **(B)**, **(C)**. Recognition of G75 and G76 was significantly different from control, (T2/NP), no peptide pulsed T2 cells (O). Recognition of G76 by OD8 and of G76 by OF-8-1 was 0.0. ($P < 0.05$) by the unpaired Student *t*-test. OVTAL-1 recognition of peptides G60 and G61 was borderline and not significantly different from controls, and was not tested for lines OD8 and OF-8-1.

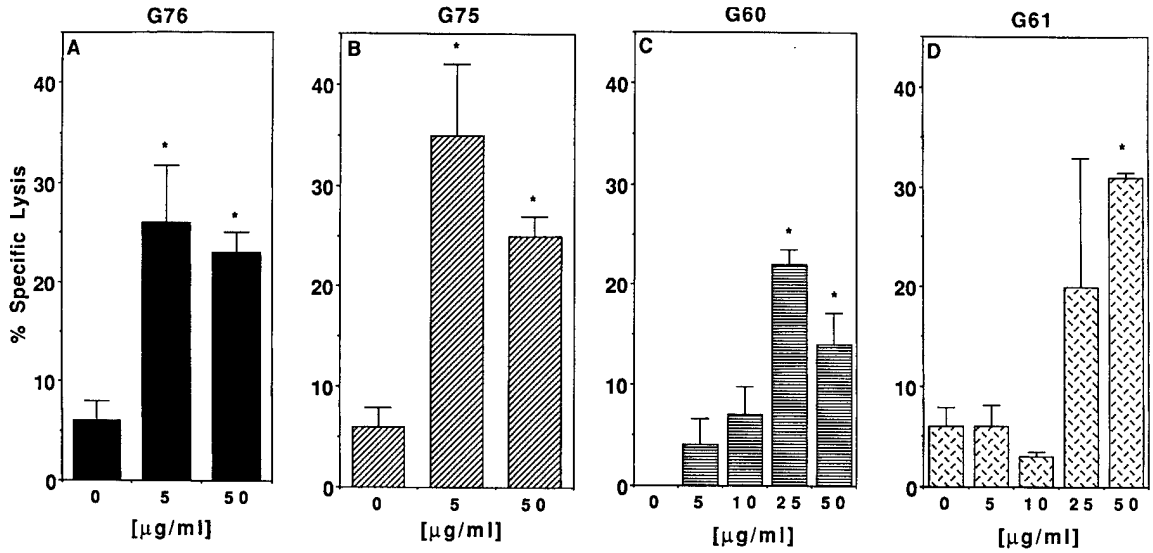

Fig. 4. Concentration dependent recognition of AES peptides G60 and G61 by OVTAL-2. T2 targets were pulsed with **(A)** G75, **(B)** G76, **(C)** G60, **(D)** G61. E:T ratio in the 5 h assay was 10:1 in all experiments. Recognition of G75, G76 and G61 was determined in the same experiment. 0, indicates T2/NP. Recognition of G60 was determined in a separate experiment performed three days later. Both G75 and G76 were recognized better than T2/NP ($P < 0.05$) at 5 μg/ml. One of two experiments performed in triplicate is shown. Results indicate mean ± SD. Recognition of G60 and G61 was significantly higher than of control (T2/NP) targets only at 25 and 50 μg/ml respectively. Experimental conditions were described in Materials and methods.

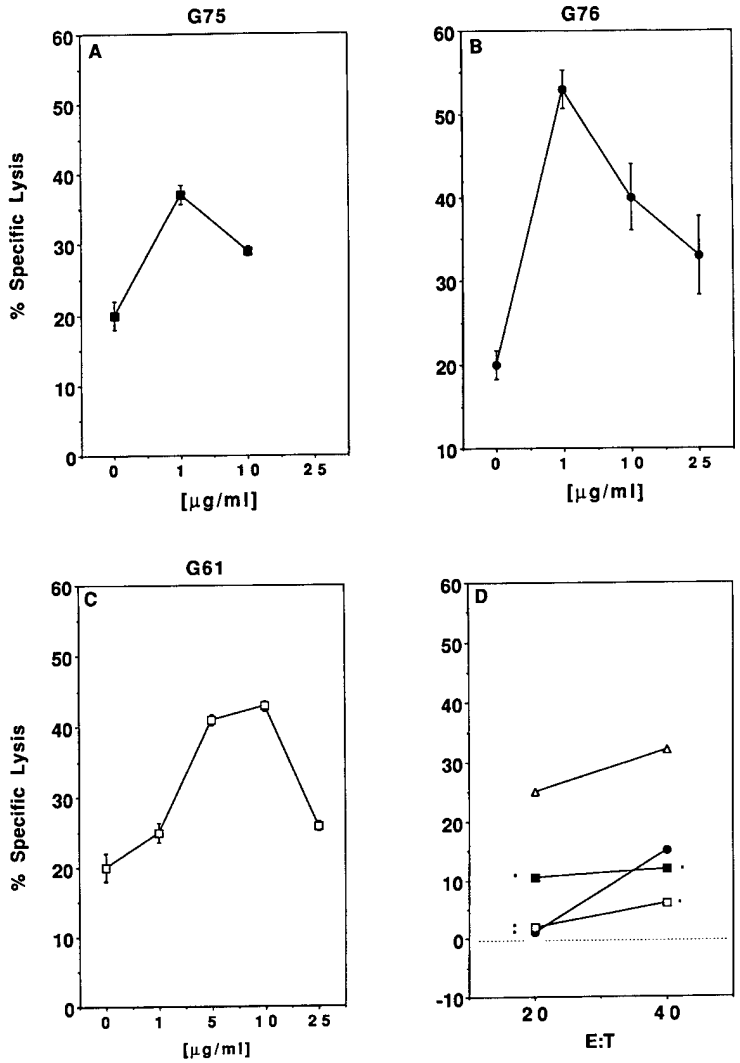

Fig. 5. **(A, B, C)** Concentration dependent recognition of AES peptides G75, G76 and G61 by BRTAL-1 in a 5 h assay. All determinations were performed in triplicate in the same experiment. Results respresent mean ± SD. E : T ratio was 20 : 1. Recognition of G75 and G76 was significantly higher than of control T2/NP (O) at 1 and 10 μg/ml. Recognition of G61 was not significantly different from control at 1 μg/ml. **(D)** Cold-target inhibition by AES peptides of the recognition of autologous tumor by BRTAL-1. All determinations were performed in the same experiment in triplicate. Results indicate mean ± SD. Because BRTAL-1 showed weak lysis of autologous tumor in 5 h assay (7.2%), the assay was continued up to 20 h to confirm the differences in inhibition of tumor lysis. T2 cells were pulsed with each peptide at 10 μg/ml. The cold : hot ratio was 10 : 1. The E : T ratio was 20 : 1 and 40 : 1. (Δ) NP, (●) G75, (■) G76, ( ) G61. (*) Significant inhibition of lysis was observed with G75, G76 and G61 at E : T ratio of 20 : 1 ($P < 0.05$) but only with G61 and G75 ($P < 0.05$), at E : T ratio of 40 : 1.

terminal area of these peptides, since all these peptides share the hexamer LPLTPL.

3.4. Cold-target inhibition experiments

To address whether AES epitopes are present on the autologous tumor with BRTAL-1, we performed cold-target inhibition experiments. All AES peptides tested inhibited recognition of autologous tumor by more than 50% compared with T2 cells pulsed with no peptide (T2/NP) at a cold : hot ratio of 10 : 1. The results in Fig. 5D also show that G76 was more effective than G75 in inhibiting recognition of the breast tumor by the autologous BR-TAL-1, suggesting that it is likely that an epitope similar to the one formed by G76 on T2 cells is expressed on autologous tumor. Autologous tumor lysis was inhibited even more efficiently than G75 and G76, by G61. It should be noted that BR-TAL-1 recognized more efficiently G75 and G76 than G61. The reasons for higher inhibition by G61 are not known, but G61 had significantly higher stabilizing ability for HLA-A2 than G75 and G76, thus it may be possible that a higher number of HLA-A2—G61 complexes are present on T2 cells over a longer period of time. These results also indicate that a peptide with similar or cross-reactive

sequence with G61 is presented by the freshly isolated metastatic breast tumor.

3.5. Both G75 and G76 specific CD8$^+$ cells are present in the freshly isolated breast TAL

The results presented above suggest that CTL specific for either G75 or G76 or both epitopes are present in the ovarian ascites and pleural effusions. To establish whether CTL specific for one of these peptides constitute a significant population, CD8$^+$ cells were isolated from a sample of breast pleural effusion (designated BRTAL-2) one week after culture in IL-2 and were further cultured using different starting numbers. When the cells in each culture were present in sufficient numbers to allow CTL assays they were tested for recognition of peptides G75 and G76. Of the 37 cultures tested, we found two cultures (15B and 27E) for which the levels of recognition of G76 were at least two-fold higher than of G75, and one culture (27F) for which the levels of recognition of G75, were at least two-fold higher than of G76 (Fig. 6A and 6B). Since these CTL were isolated from cultures initially seeded between 160 cells/well (27F, 27E) and 640 cells/well (15B), this suggests that the clonal size of G75-specific and G76-specific CTL should be significant.

To determine whether in these populations G76 specific clones are present, one of the lines, designated as 27E was recloned, and retested after expansion. The results in Fig. 6C show that the line B27E recognized G76 at both 0.1 and 1.0 μg/ml, at an E:T ratio as low as 2:1 but did not recognize G75. This confirmed the results obtained with the two ovarian TAL lines, suggesting that CD8$^+$ CTL of similar affinity and specificity for G75 and G76 are present in both epithelial tumor systems.

4. Discussion

In this study we have identified a novel candidate tumor Ag recognized by CTL present in the TAL from ovarian and breast tumors. This candidate tumor Ag consists of AES protein, which is part of the Notch complex involved in signalling for determination of cell fate during development and differentiation. The AES protein is proposed as a candidate tumor Ag based on the ability of several peptides of AES sequence to activate and inhibit the effector function of OVTAL and BRTAL.

These peptides present certain characteristics which have not been previously observed in other human peptides forming CTL epitopes:

(1) they derive from proline rich areas and contain in the sequence at least three Pro and three Leu residues;

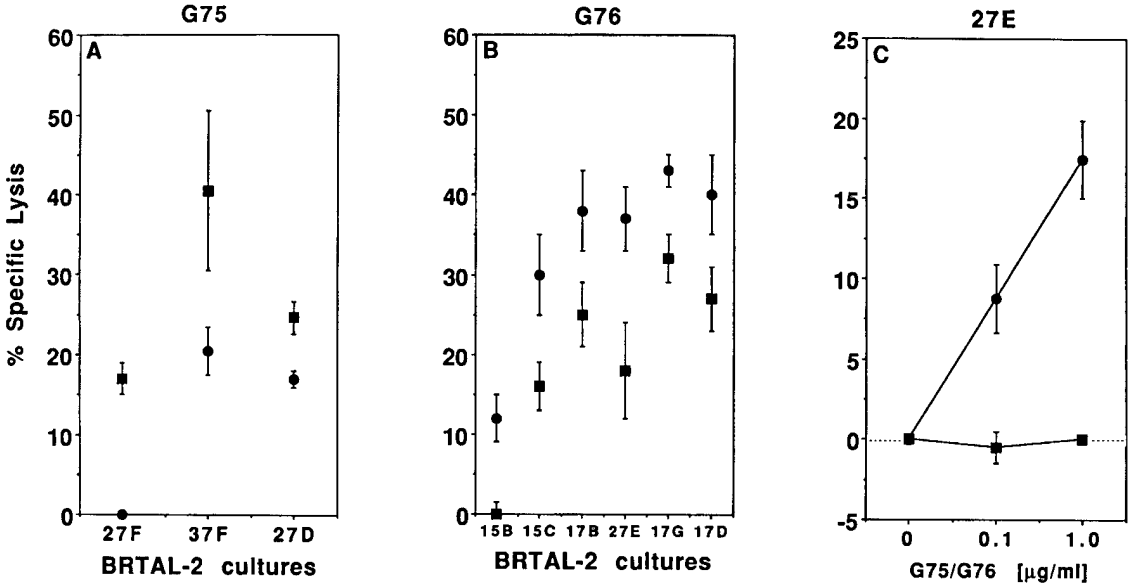

Fig. 6. **(A, B)** Patterns of G76 and G75 recognition by CD8$^+$ CTL isolated by limited cloning from BRTAL-2. **(A)** CD8$^+$ CTL cultures preferentially recognizing peptide G75. **(B)** CD8$^+$ CTL cultures preferentially recognizing G76. Target T2 cells were pulsed with 1 μg/ml of G75 and G76 respectively. Because of the large number of cultures to be tested for each culture, recognition of G75 (■) or G76 (●) was determined by an initial screening in the same experiment in duplicate. All resulting cultures were tested twice for recognition of G75 and G76. Only cultures which were confirmed in both experiments to preferentially recognize G75 or G76 were considered positive. The E:T ratio was 3:1. CD8$^+$ cells in the wells indicated as 5, 6, 7, 8 were initially seeded at 640, 320, 160 and 80 cells/well, respectively. Designations e.g. 27F indicate plate, column, row **(C)** Concentration- dependent recognition of G76 by line B27E isolated from a CD8$^+$ culture previously found in a separate experiment **(B)** to preferentially recognize G76 over G75. The E:T ratio was 2:1. (■) G75, (●) G76; **(C)** Recognition of G76 was significant compared with that of G75 at both 0.1 and 1.0 μg/ml ($P < 0.05$). The results show the means and standard deviations of one CTL assay performed in triplicate.

(2) two of these active peptides contain Pro at position 2 (P2). P2 corresponds to the main anchor position for peptide binding to HLA-A2. It is generally occupied by Leu/Ile and less frequently by Met, Ala, and Thr (Hunt et al., 1992);
(3) the most active peptides containing Pro at P2 were generally recognized with higher affinity, at 10-fold lower concentrations than the corresponding overlapping nonamers (AL)PLTPLPV containing canonical Leu (P2) and binding HLA-A2 with high affinity.
(4) One of the active peptides recognized with high affinity by ovarian and breast CTL, G76 corresponds to a fragment of a likely variant AES protein. This epitope is characterized by a Leu- > Gly change in the N-terminal residue, a change unlikely to be the result of a point mutation in the Leu codon, since no point mutations in the Leu codon can lead to the Gly codon (Stifani, S., pers. comm.).

In determining the recognition of the candidate natural epitope G76, we noted that its recognition peaked at 1–5 μg/ml and was inhibited at higher concentrations (25–50 μg/ml). This may reflect sometimes the presence of impurities in the HPLC purified peptides. For Pro-rich proteins, this may also reflect Ag aggregation/dimerization; This inhibitory activity was observed, at the same concentrations with peptide G75 which differs from G76 only at the N-terminal Leu. Although these results are preliminary since they were obtained with a small panel of effectors, they indicate that existing CTL recognize G75 and mainly G76 when pulsed on T2 targets at concentrations as low as 0.2–0.5 μg/ml (range 250–600 nM). These concentrations are significantly lower than the concentrations required to sensitize targets by most other peptides recognized by CTL in epithelial cancers (Fisk et al., 1995) and the majority of the melanoma tumor Ag (Houghton, 1994). Therefore, the possibility that G76 specific CTL are high affinity clones which are inhibited by high Ag concentrations, in a similar way with CTL recognizing classic Ag (Alexander-Miller et al., 1996) may deserve further investigation since it may provide a mechanism for tumor escape.

Based on cold-target inhibition studies, we surmise, that complexes structurally similar to the one formed by the AES peptides are present on the surface of tumor cells. Ongoing studies are attempting to clarify whether all three AES peptides are simultaneously presented by the tumor and the implications of Ag presentation from peptides containing Pro at P2.

AES peptide G76:GPLTPLPV was identified after MS sequencing of an ion with m/z 793 present in a HPLC fraction of peptides acid-eluted from immunoaffinity separated HLA-A2 molecules from a tumor line. This fraction reconstituted the CTL effector function of two OVTAL lines (Fisk et al., 1997a,b). The signal intensity of the ion 793 in two consecutive HPLC fractions matched the CTL activity induced by these fractions (Fisk et al., 1997b), thus it was considered a primary candidate for sequencing. Although our results neither provide certitude that ion 793 is a peptide, nor proof that it is the only active component of the peak 793, the approach we used may be useful for other Ag characterization studies. The extensive handling of samples used for Ag identification is accompanied by substantial losses in material (sometimes up to 90%) (Udaka et al., 1982), which need to be compensated by growing higher numbers of cells. The availability of small amounts of samples for peptide sequencing, does not allow a direct answer to the questions as to whether a particular ion present in an active peak has attached other groups (i.e. phospholipids, sugars) or even shorter peptides that may be active by themselves. In contrast, the focus on a candidate peptide and analysis of recognition of overlapping synthetic peptides may provide a rapid answer to the question whether a candidate tumor Ag, is recognized i.e. is/was immunogenic in vivo.

The critical tests of tumor Ag identification strategy are whether: (a) the candidate peptide is active in inducing effector function by T cells from the tumor environment and (b) the gene and its corresponding source protein are expressed by the tumor, i.e. CTL do not recognize cross-reactive species on other targets. Our results show that the AES peptides meet the criterion of activating of effector function. Ongoing studies in our laboratory have also found that peptides G75, and G76 but not G60 can induce IL-2 secretion by OVTAL, within 24h, suggesting that they can activate additional signal transduction pathways in effectors (Babcock et al., Manuscript in preparation). Furthermore, preliminary studies in our laboratory could not unambigously establish that (a) peptides with the group VP instead of PV: G59, G58, G59 and (b) peptides with extended C-terminal: G77, G78, G58, G59 are recognized by BRTAL and OVTAL. Thus, additional studies are needed to clarify these points. In the absence, at this time, of antibodies that unequivocally recognize human AES-1/2 in tumors and healthy tissues, we cannot quantitate the AES levels. Preliminary results in our laboratory using PCR and primers and probes specific for AES-1,2 indicate that AES transcripts are present in both the ovarian SKOV3.A2 and breast SKBR3.A2 lines, and they are distinct from the transcripts of the TLE-1-4 proteins. However, since the presence of a transcript does not always indicate the abundance of a protein, additional studies are needed to address this point.

The AES genes (also designated Grg) mapped to human chromosome 19 (Mallo et al., 1995), are part of the Enhancer of split [E(spl)] complex of genes, that also includes the similar TLE genes (Stifani et al., 1992). The E(spl) genes form a neurogenic locus in *Drosophila* (Artavanis-Tsakonas et al., 1995). The exact function of E(spl) proteins is not known, but it is possible that they function

as transcriptional repressors of effector genes for cell differentiation (Jarriault et al., 1995). These repressor pathways are induced by the activation of the membrane-bound Notch receptor and signal the suppression of differentiation. It was recently reported that TLE family members are overexpressed in combinatorial ways during differentiation of mouse embryonic carcinoma cells in vitro (Yao et al., 1998). The role of AES and its involvement in carcinogenesis or maintenance of undifferentiated state is unknown but the mouse analog Grg was implicated in inhibition of gene transcription (Mallo et al., 1995b). The AES proteins may be widely distributed in adult mice tissues (Mallo et al., 1995a) and possibly in human tissues (Miyasaka et al., 1993).

Four mammalian *Notch* genes (1, 2, 3, 4) have been identified (Shirayoshi et al., 1997). They are highly conserved relative to the Drosophila *Notch* gene and appear to be important for cell differentiation and neoplasia associated translocation (Larsson et al., 1994). Expression of *Notch*-1/*Notch*-2 proteins have been reported in adult tissues of the mouse including among others brain, thymus, spleen and lung (Ellisen et al., 1991), as well as human bone marrow $CD34^+$ stem cells (Varnum-Finney et al., 1998). Transcripts of human *Notch*-1 are abundant in human fetal tissues while overexpression or truncation of Notch-(Tan 1) are important determinants of oncotropic activity (Ellisen et al., 1991; Pear et al., 1996).

It may be tempting to propose that: (a) the synthesis of AES proteins also increase in cancer cells, to maintain the undifferentiated state as reported for TLE genes (Liu et al., 1995); (b) AES may be subjected to a more rapid turnover and/or interacts with another protein as recently reported for TLE proteins (Palaparti et al., 1997) and/or is misprocessed. (c) Overexpression of Notch-1 and Notch-2, as well as of the proteins of the TLE complex may result in overexpression and misprocessing of AES proteins. All these possibilities have been reported to lead to CTL epitope formation (Yewdell et al., 1996; Michalek et al., 1993). It remains to be seen whether AES is expressed in normal tissues, and whether the CTL recognizing these peptides also lyse healthy tissues. If the origin of ion 793 as well as the wild-type AES CTL epitopes will be confirmed, this may provide a novel Ag to target in cancer vaccination studies.

Acknowledgments

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ORIGINAL ARTICLE

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HER-2/*neu* peptide specificity in the recognition of HLA-A2 by natural killer cells

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Abstract Although natural killer (NK) cells have been described as non-MHC-restricted, new evidence suggests that NK activity can be either up- or down-regulated after interaction with the peptide–MHC-class-I complex expressed on target cells. However, the epitope(s) recognized by NK cells have remained ill-defined. We investigated NK cell recognition of synthetic peptides representing a portion of a self-protein encoded by the HER-2/*neu* (HER-2) proto-oncogene and presented by HLA-A2. HER-2 nonapeptides C85, E89, and E75 were found partially to protect T2 targets from lysis by freshly isolated and interleukin-2(IL-2)-activated NK cells (either HLA-A2$^+$ or A2$^-$). This inhibition was not solely due to changes in the level of HLA-A2 expression or conformation of serological HLA-A2 epitopes. Using single-amino-acid variants at position 1 (P1) of two HER-2 peptides, we observed that protection of targets was dependent on the sequence and the side-chain. These results suggest similarities in the mechanism of target recognition by NK and T cells. This information may be important for understanding the mechanisms of tumor escape from immunosurveillance and could help explain the aggressiveness of HER-2-overexpressing tumor cells.

Key words Natural killer cells · HER-2/*neu* · Peptides · MHC · Tumor immunity

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Introduction

Natural killer (NK) cells are thought to play an important role in the elimination of virus-infected cells and cancer cells [5, 26, 39]. Although target-cell killing by NK cells has traditionally been described as non-MHC-restricted, interaction of NK-cell-inhibitory receptors with MHC class I molecules often leads to a down-regulation of NK cytolytic function in proportion to the level of MHC class I expression on the targets [20, 27, 40, 42]. Recent reports also indicated that single amino acid mutations within the peptide-binding groove of the MHC molecule can affect target cell sensitivity to lysis, suggesting that NK cells recognize different conformations induced by peptides bound in the MHC class I pockets [19, 37]. This hypothesis has been supported by observations that external loading of target cells with either self or foreign peptides can enhance or inhibit sensitivity to NK-mediated lysis in a peptide-specific manner independent of the level of MHC class I up-regulation [7, 25, 28, 38]. However, the basis for peptide specificity in the induction of lysis or protection is unknown. Further analysis of the mechanism of NK recognition of peptides may provide an important insight into the function of NK cell specificity for tumor cells.

The HER-2/*neu* (HER-2) proto-oncogene product is overexpressed in a variety of human cancers including breast, ovarian, colon, lung, and stomach, and its overexpression by breast and ovarian cancers has been shown to correlate with earlier relapse and a worse prognosis [36]. Since it has been reported that HER-2 overexpression also correlates with decreased NK cell activity [41], we wanted to determine if HER-2 peptides are directly involved in NK cell inhibition. Therefore, we used HER-2 peptides recognized by cytotoxic T lymphocytes (CTL) as targets, where the question of the sequence specificity in NK recognition can be addressed. In this report we investigated the ability of freshly isolated and in vitro interleukin-2(IL-2)-activated NK cells to recognize self-HER-2 peptides that bind to the HLA-

A2 molecule with variable affinities and induce conformational changes in the α1 and α2 domains. We found that these peptides decreased NK-mediated lysis of T2 cells, and the ability to inhibit lysis depended more upon peptide sequence than the ability to up-regulate or induce conformational changes of HLA-A2 on the target cells. Interestingly, the peptide that induced the most HLA-A2 up-regulation and conformational changes inhibited lysis least. Furthermore, targets pulsed with HER-2 peptide variants containing amino acid substitutions at position 1 (P1) showed either side-chain-dependent protection or increased sensitivity to NK-mediated lysis. Again, increased levels of lysis inhibition among the peptides did not correlate with increased levels of expression of HLA-A2, as detected by the W6/32 mAb specific for a monomorphic MHC I epitope (α3 domain), and lysis inhibition did not correlate with conformational changes of HLA-A2 detected by the MA2.1 mAb (α1 domain). Peptides that induced the most change in expression and conformation of HLA-A2 were often less effective at inhibiting lysis. However, the enhanced sensitivity to NK lysis seen with one peptide was paralleled by changes in the conformational epitope recognized by the BB7.2 mAb (α2 domain). These results indicate an important effect of changes in peptide sequence at position 1, and provide further evidence that the mechanism of NK target recognition has some similarity to that of T cells in that it is determined by interactions of peptide side-chains with NK receptors. These findings may also be helpful in explaining why cancer patients with tumors overexpressing HER-2 have a worse prognosis.

Materials and methods

Target cells

The T2 line has been described previously [15] and was a generous gift from Dr. Peter Cresswell (Yale Univeristy School of Medicine, New Haven, Conn.). The B cell line C1R:A2, an HLA-A2-gene-transfected derivative of C1R, was a gift from Dr. William Biddision (National Institute of Neurological Disorders, Bethesda, Md.). C1R:A2 cells were transfected with the plasmid pCMV.HER-2 encoding a full-length HER-2 cDNA (the kind gift of Dr. Mien-Chie Hung, Department of Tumor Biology, M.D. Anderson Cancer Center). C1R:A2:HER-2 transfectants were selected by resistance to hygromycin B by co-transfection of SV2.Hygro plasmids (ATCC, Rockville, Md.).

Effector cells

Peripheral blood buffy coats of normal donors were purchased from a local blood center, and mononuclear cells (PBMC) prepared by Ficoll-Hypaque gradient separation [24]. NK cells were enriched to high purity by negative selection using a MACS NK Isolation Kit (Miltenyi Biotec, Auburn, Calilf.). In brief, PBMC were incubated for 15 min at 4 °C with a cocktail of monoclonal antibodies (mAb) recognizing CD3, CD4, CD19, and CD33, washed, and then incubated for an additional 15 min with colloidal superparamagnetic microbead-labelled antibody reacting to the primary antibodies (Beckton Dickinson, Mountain View, Calif.). The cells were then passed twice through an iron-wool column placed within a strong magnetic field, and the nonadherent cells collected. The effluent population was routinely 91.7%–98.2% $CD56^+$, $CD3^-$ NK cells, 0.1%–1.4% $CD56^+$, $CD3^+$ T cells, and 0.2%–1.3% $CD56^-$, $CD3^+$ T cells as determined by two-color flow cytometry [35].

For IL-2 activation, NK cells were cultured for 5–7 days in RPMI-1640 medium supplemented with 10 mM HEPES buffer, 10% human AB serum, antibiotics, 2 mM glutamine, 2 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 μM 2-mercaptoethanol (complete RPMI medium), and 500 U/ml highly purified human recombinant rIL-2 (18×10^6 IU/mg; Cetus Corp., Emeryville, Calif.). The NK cell line, NKL (kindly provided by Dr. M.J. Robertson, Dana Farber Cancer Institute, Boston, Mass.) was obtained from peripheral blood of a patient with a $CD3^-$, $CD16^+$, $CD56^+$ large granular lymphoproliferative disorder [18]. These cells were maintained in culture in complete RPMI medium supplemented with 30 U/ml IL-2.

In some experiments, the CTL line 41 (CTL-41) was used as a source of effectors. This line was developed by repeated in vitro stimulation of $HLA\text{-}A2^+$ peripheral blood mononuclear cells from a healthy donor with peptide C84: HER-2 (971–979 V) and a longer peptide C43: HER-2 (968–981) [12]. For these studies, CTL-41 were maintained in culture with monthly restimulation with 10 μg/ml C84 peptide and autologous or allogeneic $HLA\text{-}A2^+$ PBMC. The CTL used as effectors were selected on mAb-coated plates (AIS Micro CELLector, Applied Immune Sciences, Menlo Park, Calif.), and were $CD3^+$, $CD4^-$, $CD8^+$. Clones were isolated from the CTL-41 line, as previously described [14].

Synthetic peptides

Synthetic peptides corresponding to sequences in HER-2: E75 (369–377), E89 (851–859), C85 (971–979), and recognized by ovarian tumor-specific CTL, have been reported previously [12–14]. The amino acid sequences of these peptides are shown in Table 1. Variants of the C85 peptide substituted at P1 are designated as G1, F1, T1, and K1 [12]. The E75 peptide substituted at P is designated as peptide F41. The synthetic peptides used in this study were prepared by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center, purified to 92%–95% by HPLC, and dissolved in phosphate-buffered saline (PBS) at a stock concentration of 1 mg/ml.

Table 1 Sequences of synthetic HER-2 peptides

Code	Position	1	2	3	4	5	6	7	8	9
E75	369–377	K	I	F	G	S	L	A	F	L
F41		G	–	–	–	–	–	–	–	–
E89	851–859	V	L	V	K	S	P	N	H	V
C85	971–979	E	L	V	S	E	F	S	R	M
G1		G	–	–	–	–	–	–	–	–
F1		F	–	–	–	–	–	–	–	–
T1		T	–	–	–	–	–	–	–	–
K1		K	–	–	–	–	–	–	–	–
C84		E	–	–	–	–	–	–	–	✓

Cytotoxicity assay

The ^{51}Cr release assay has been described in detail previously [24]. For peptide-pulsing experiments, ^{51}Cr-labelled T2 cells were dispensed into 96-well microtiter plates and preincubated for 2 h in serum-free RPMI medium, to which was added either 10 µl peptide (100 µg/ml final concentration), or an equivalent volume of PBS as a control. Effector cells, suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum, were then added in various E:T ratios (ranging from 40:1 to 1:1), and the culture supernatants were tested for chromium release after 4–5 h of culture. Each experimental condition was tested in triplicate. Results are expressed as the percentage specific lysis according to the formula $(E - S)/(M - S) \times 100$, where E is the radioactivity (cpm) of experimental wells containing both effectors and targets, S is the spontaneous release of ^{51}Cr from targets incubated in medium (with and without peptide), and M represents the radioactivity for targets incubated with 0.2% Triton X-100 (maximum release). In some experiments the cytotoxicity was expressed as lytic units (LU), where 1 LU is the number of effector cells required for lysis of 30% of the target cells [35]; when this calculation is used, the results are expressed as $LU_{30}/10^6$ effector cells.

In studies designed to analyze the sensitivity of HER-2-gene-transfected cell lines to lysis, NK or CTL effector cells were incubated for 4–5 h with ^{51}Cr-labelled CIR:A2:HER-2-transfected target cells at effector-to-target ratios (E:T) ranging from 12:1 to 50:1

Flow-cytometric analysis

Expression of HLA-A2 on T2 target cells was evaluated by flow cytometry, using BB7.2, MA2.1 and W6/32 mAb. W6/32 mAb (Dako, Dakopatts, Denmark) recognizes a monomorphic epitope common to HLA-A, -B, and -C. The anti-HLA-A2 mAb, BB7.2 (mouse IgG2b) and MA2.1 (mouse IgG1) were obtained from the American Type Culture Collection (ATCC). Other antibodies used in this study included anti-CD11a, anti-CD18, anti-CD58, and anti-CD56 (Beckton-Dickinson, Mountain View, Calif.); and Ab2, reacting with the extracellular domain of HER-2 protein (Oncogene Science, Uniondale, New York). Briefly, 5×10^5 cells were incubated for 30 min at 4 °C with primary antibody (or an isotype control antibody nonreactive with human cells), washed, and then incubated for an additional 30 min with fluorescein-isothiocyanate-conjugated goat anti-(mouse Ig). Flow-cytometric analyses were performed on 5000 gated events/sample, using a FACScan flow cytometer (Becton-Dickinson, Mountain View, Calif.) and Consort 30 software.

To analyze the effect of peptide pulsing on HLA-A2 expression, T2 cells were incubated for 2 h at 37 °C with 10–100 µg/ml peptide (or PBS alone as a control), prior to labelling with the primary mAb. All cells tested were positive for HLA-A2 expression; data are reported as the mean channel fluorescence, indicative of the channel number corresponding to the average peak of fluorescence [6, 31, 35].

Statistical analysis

The data were analyzed statistically using Prism 2.01 software (GraphPad Prism for Scientists, Sorrento, Calif.). Multiple groups were compared by the Newman Keuls one-way analysis of variance. When only two groups were compared, Student's t-test was used. Differences were considered significant when P was less than 0.05.

Results

HER-2 peptides inhibit NK-mediated lysis of T2 cells

In the first series of experiments, we investigated the effects of HER-2 self-peptides on the sensitivity of T2 target cells to lysis by NK cells. The T2 cells have a defect in TAP (transporter-associated with antigen presentation) proteins and display "empty" HLA-A2 molecules [33] that can be loaded exogenously with peptides having the proper anchors for binding to HLA-A2, i.e. L/M/I/V (P2) and V/L/M/I (P9). For our studies we used three different synthetic nonapeptides of HER-2 that display these HLA-A2 anchors: E75, E89 and C85 (the amino acid sequences of these peptides are shown in Table 1). These peptides were previously found to reconstitute recognition of $CD8^+$, $CD4^-$ CTL lines derived from ovarian tumor-associated lymphocytes [12], suggesting that HER-2 is naturally processed into identical or similar peptides presented by HLA-A2 on tumor cells.

The T2 targets were pulsed with peptides at a concentration of 100 µg/ml prior to addition of effector cells. In agreement with others [32], we observed that untreated T2 targets were sensitive to lysis by freshly isolated and IL-2-activated peripheral blood NK cells from all healthy donors tested (Fig. 1). However, T2 cells pulsed with HER-2 peptides were significantly protected from killing by unstimulated HLA-$A2^+$ and HLA-$A2^-$ NK cells (Fig. 1A, B). These peptides also protected T2 targets from lysis by IL-2-activated HLA-$A2^+$ and HLA-$A2^-$ NK cells (Fig. 1C, D). The results from four representative donors of eight tested are shown in Fig. 1. The reduction in lysis of peptide-pulsed

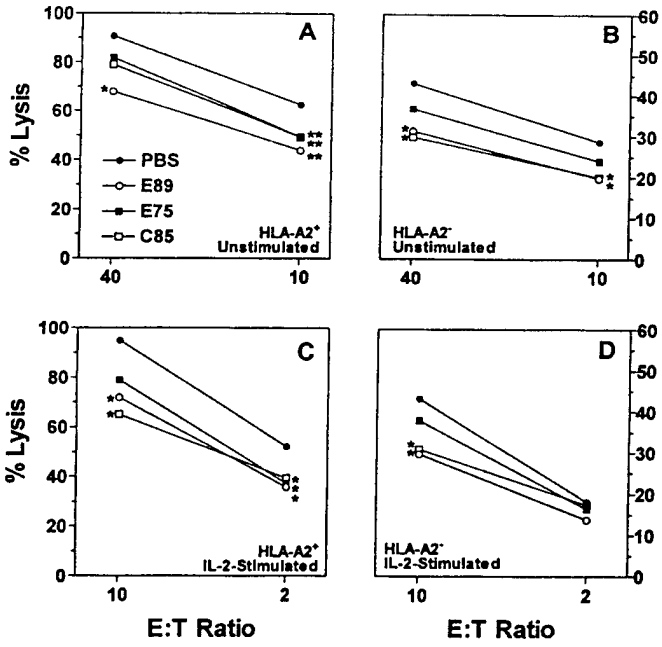

Fig. 1A–D Recognition of HER-2 peptide-pulsed T2 cells by natural killer (NK) cells. Magnetically sorted NK cells from four healthy donors (one per panel) were tested for lytic activity against HER-2-peptide-pulsed T2 cells in a ^{51}Cr-release assay. The NK cells were (A) unstimulated HLA-$A2^+$, (B) unstimulated HLA-$A2^-$, (C) interleukin-2(*IL-2*)-stimulated HLA-$A2^+$, and (D) IL-2-stimulated HLA-$A2^-$. Significant inhibition of lysis: $^*P < 0.05$ compared to phosphate-buffered saline (*PBS*) control; $^{**}P < 0.01$ compared to PBS control

target cells was consistently observed at multiple E:T ratios and ranged from 15% to 30%.

Because sensitivity of target cells to NK-mediated lysis has been shown to be inversely related to the levels of MHC class I expression [40], we next determined whether the resistance of HER-2-pulsed T2 targets to lysis by NK cells was associated with an increase in MHC class I molecules caused by peptide-induced stabilization [6]. As shown in Fig. 2, T2 cells incubated with HER-2 peptides displayed an increase in the relative density of surface HLA class I molecules as detected by the HLA-A, -B, -C-specific W6/32 mAb; this was observed as an increase in the fluorescence intensity of mAb-labelled peptide-pulsed T2 cells compared to controls. However, there were marked differences among the peptides in the relative density of class I molecules induced, with an approximately twofold and threefold increase caused by E89 and E75 respectively. The C85 peptide only slightly increased MHC class I expression. Despite these large differences in MHC class I expression, the level of protection afforded by E89 and C85 peptides was comparable. Although E75 increased MHC expression the most, it was consistently least effective at inhibiting target lysis (Fig. 1). These results indicate that the increased resistance of T2 to lysis by NK cells induced by C85 was not simply caused by up-regulation of MHC class I molecules.

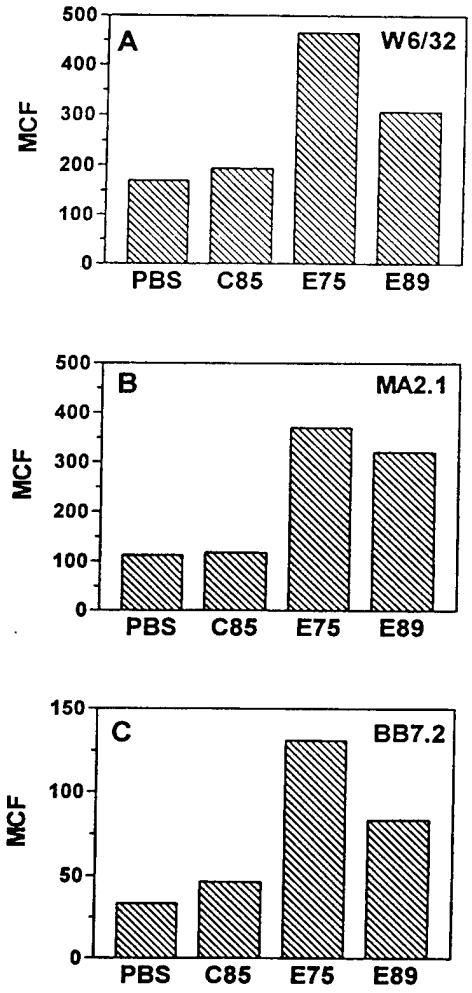

Fig. 2A–C Up-regulation of HLA-A2 expression by HER-2 peptides. T2 cells pulsed with or without (PBS control) HER-2 peptides, were analyzed by flow cytometry for expression of epitopes recognized by the W6/32, MA2.1, and BB7.2 mAb. *Bars* the mean channel fluorescence (*MCF*) value, i.e., the channel corresponding to the mean fluorescence intensity of positively stained cells

To determine if peptide-induced protection was related to changes in the conformation of HLA-A2 molecules, we also analyzed peptide-pulsed and control T2 cells for expression of conformational epitopes recognized by BB7.2 and MA2.1 mAb. The epitope recognized by the BB7.2 mAb is located on the N-terminal loop of the α2 domain (including W108) of HLA-A2, in an area not expected to contact the peptide directly [34]. MA2.1 mAb reacts with the α1 domain of HLA-A2 at residues 64–68, which border the A and B pockets of the peptide-binding groove; mutations of HLA-A2 in this area have been reported to affect T cell recognition significantly [16, 34]. The results in Fig. 2 indicate that the decrease in the sensitivity of T2 to lysis by NK cells, after pulsing with a particular peptide, was not proportional to the increase in the expression of either of these conformational epitopes. On the contrary, while MA2.1 and BB7.2 epitopes were expressed at approximately threefold higher levels on E75-pulsed T2 cells compared to untreated or C85-pulsed targets, the protection induced by E75 was, in most cases, less than that of the other peptides (Fig. 1).

Recognition of peptide variants by NK cells

Crystallography studies have shown that the N-terminal (P1) residue of peptides binds within the A pocket of the MHC molecule, and that the nature of the side-chain of this residue affects peptide binding to HLA-A2 [3]. To address the question of whether a single amino acid substitution at P1 would alter the ability of a HER-2 peptide to protect targets from NK-mediated lysis, we created a series of C85 variants by replacing the glutanic acid at P1 with lysine (variant K1), glycine (variant G1), threonine (variant T1) or phenylalanine (variant F1) (Table 1). These peptide variants do not have changes in the dominant anchors for HLA-A2 at P2 (L/M/I/V) and P9 (V/L/M/I), so they should still bind to HLA-A2. Using a peptide concentration that was protective for C85, we tested the ability of the peptide variants to protect T2 cells from NK-mediated lysis (Fig. 3A). Significant differences between the peptides were observed regarding their ability to affect T2 lysis. Specifically, we found that the K1 variant was as effective as the natural C85 peptide in protecting T2 cells from lysis, while the F1 and G1 variants did not significantly inhibit T2 lysis compared to control T2 cells treated with PBS. In contrast, T2 targets pulsed with the T1 variant not

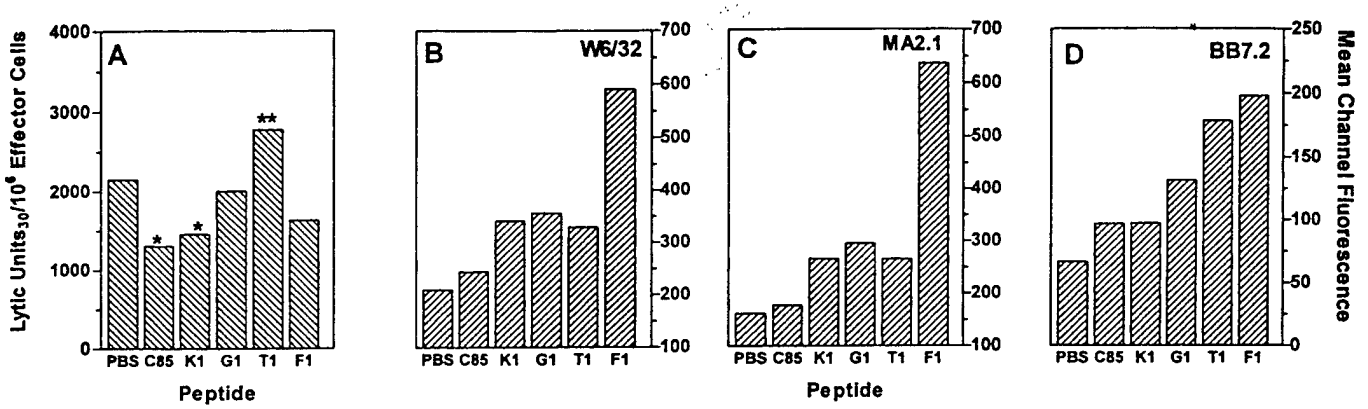

Fig. 3A–D Recognition of HER-2 peptide variants by NK cells. A Immunomagnetically isolated HLA-A2$^+$ NK cells were tested for cytolytic activity against T2 targets pulsed with 100 μg/ml C85 and C85 variants, and the results from four separate donors were averaged. Data are expressed as lytic units as described in Materials and methods. * There was significant protection of T2 after pulsing with C85 (E1) or the K1 variant ($P < 0.05$ compared to PBS-treated control T2 targets). ** The T1 variant caused significant enhancement of T2 lysis compared to both C85-pulsed and control targets ($P < 0.05$). B–D Three different mAb (W6/32, MA2.1, and BB7.2) were used to detect MHC class I expression by T2 cells pulsed with the same peptides used in A

only were not protected, but instead were even more susceptible to lysis by NK cells than were PBS-treated controls. On the basis of a comparison of cytotoxicity (LU), they were also twofold more susceptible to NK lysis than C85-pulsed T2.

All of these C85 variants up-regulated and stabilized MHC class I expression as detected by the W6/32 mAb, albeit to different degrees (Fig. 3B). The conformational epitopes recognized by MA2.1 and BB7.2 mAb were also up-regulated when compared to PBS-treated T2 targets (Fig. 3C, D). The levels of expression of W6/32 and MA2.1 HLA-A2 epitopes on T2 cells pulsed with K1, G1, and T1 variants were similar, and they were higher than the levels induced by C85. The F1 variant induced a twofold higher increase in these epitopes relative to the other variants. The T1 and F1 variants induced the highest levels of BB7.2 epitope expression among the variants tested.

When the levels of expression of MHC class I and the BB7.2 and MA2.1 conformational epitopes were compared to protection from lysis, the ability of a particular peptide to down-regulate target sensitivity to NK lysis was not directly proportional to the increase in the level of HLA-A2 expression. For example, neither the G1 nor F1 variant was significantly protective, even though F1 induced a substantially higher expression of W6/32, MA2.1, and BB7.1 epitopes. Furthermore, compared to C85, the T1 peptide enhanced the susceptibility of T2 cells to NK-mediated lysis, even though this variant induced higher levels of HLA-A2 expression and conformational changes. The K1 variant was as protective as C85, but induced higher levels of MHC class I than did the natural peptide, as detected by the W6/32 and MA2.1 mAb. Again, these data support the observation that increased MHC class I does not always correlate with enhanced resistance to lysis by NK cells.

The results in Fig. 3 show a significant role for the P1 residue side-chain in NK inhibition. Protective peptides in this study (C85 and K1) have charged side-chains, while the nonprotective peptides (F1 and G1) have nonpolar side-chains. T1, which enhanced NK sensitivity, has a hydroxyl group. To confirm that the NK inhibition is dependent on the side-chain of the amino acid at P1, we investigated NK recognition of T2 cells pulsed with the weakly NK protective peptide E75 and its P1 variant, F41. In the latter variant, the lysine at P1 in the natural peptide is substituted with glycine, which lacks a side-chain (Table 1). This K → G change at P1 of E75 is identical to the change between the K1 and G1 variants of C85 described above. Thus F41 was expected to be less protective than E75. We observed that both E75 and F41 showed a similar concentration-dependent ability to up-regulate HLA-A2 expression on T2 cells: for example, at 100 μg/ml, the mean channel fluorescence for the expression of the BB7.2 epitope was approximately threefold higher for both E75-pulsed and F41-pulsed targets than for the controls (data not shown). Both peptides also protected T2 targets from lysis by the NK cell line (NKL) when used at concentrations of 10–100 μg/ml (Fig. 4A). However, at 200 μg/ml, E75 (charged P1 side chain) was significantly more protective ($P < 0.001$) against NK lysis than was F41 (nonpolar P1 side-chain), supporting the conclusion from the previous experiment.

To rule out the possibility that the HER-2 peptides were stabilizing non-HLA-A2 MHC or nonclassical MHC molecules that could mediate a decrease in NK sensitivity [1, 2], E75-pulsed T2 targets were treated with either the HLA-A2-specific MA2.1 mAb or control antibody before lysis by the NK cell line was assessed. As seen in Fig. 4B, HER-2-peptide-induced inhibition of lysis was most likely mediated directly through interaction with HLA-A2, because MA2.1 antibody completely blocked the inhibition of T2 lysis at 100 μg/ml E75 ($P < 0.05$) and significantly blocked the higher inhibition at 200 μg/ml E75 ($P < 0.01$).

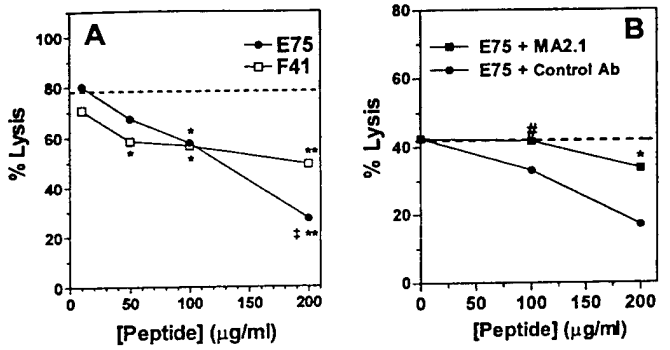

Fig. 4A, B Protection of T2 targets by HER-2 peptides is concentration-dependent and HLA-A2-dependent. T2 cells pulsed with E75 or its variant, F41, were tested for susceptibility to lysis by the HLA-A2$^+$ NK cell line, NKL, at an E:T ratio of 40:1. - - - - The percentage lysis of PBS-treated control T2 targets (78% for **A** and 42% for **B**). **A** Significant protection from lysis: * $P < 0.01$ compared to PBS-treated control; ** $P < 0.001$ compared to PBS control; ‡ E75 inhibited lysis significantly better than F41 at 200 μg/ml ($P < 0.001$). **B** E75-pulsed T2 targets were incubated with HLA-A2-specific blocking antibody (MA2.1) or control antibody. # MA2.1 completely blocked the protection from lysis at 100 μg/ml E75 ($P < 0.05$). * Significant blocking of the protection from lysis also occurred at 200 μg/ml E75 ($P < 0.01$)

Recognition of HER-2-transfected C1R:A2 cells by NK cells

Under physiological conditions, NK cells interact with HLA-A2 molecules presenting peptides processed endogenously. Therefore, it was of interest to determine if endogenously processed HER-2 peptides could also protect target cells from lysis by NK cells. We approached this question using C1R:A2 cells transfected with the HER-2 gene (C1R:A2:HER-2$^+$ cells). C1R:A2:HER-2$^+$ cells were cloned by stringent limiting dilution, and three clones (HER-2:G, HER-2:B, and HER-2:L), expressing different levels of surface HER-2, were tested for sensitivity to lysis by CTL and NK cells. CTL clones (CD3$^+$, CD8$^+$, CD4$^-$) were developed by in vitro stimulation of HLA-A2$^+$ mononuclear cells from a healthy donor with C84, a P9-substituted (M → V) C85 peptide [12]. As shown in Fig. 5, two clones (2C2 and 2C3) recognized the C1R:A2:HER-2:L clone but not C1R:A2 cells lacking HER-2 gene expression, suggesting that an epitope similar to C85 was presented by C1R:A2:HER-2$^+$ cells. However, the HER-2$^+$ clones were more resistant to lysis by IL-2-stimulated NK cells than were the nontransfected

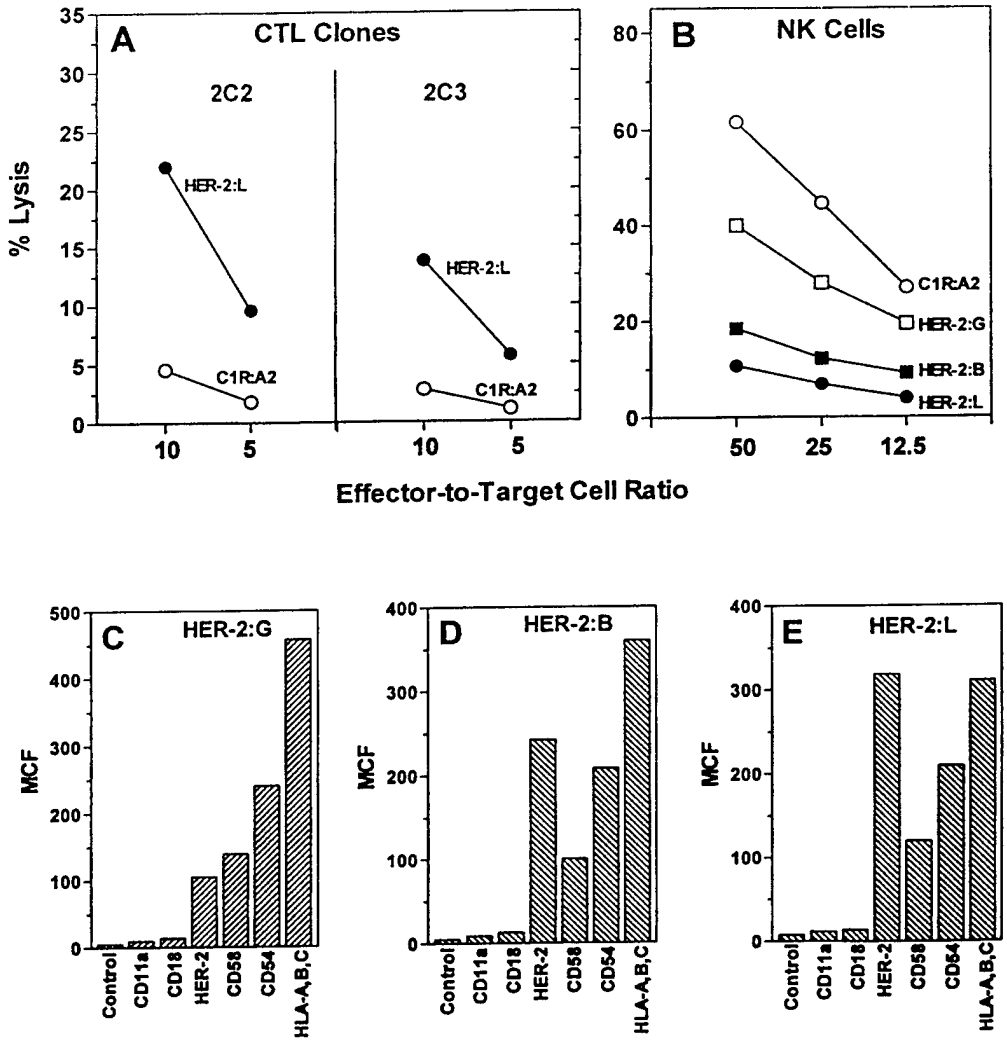

Fig. 5A–E Lysis of the HER-2 gene-transfected target cells by cytotoxic T lymphocytes (CTL) and NK cells. **A, B** Nontransfected and HER-2-transfected C1R:A2 cells (HER-2:G, HER-2:B, and HER-2:L) were tested for susceptibility to lysis by two C85-specific HLA-A2$^+$ CTL clones (2C2 and 2C3) and magnetically sorted, IL-2-activated HLA-A2$^+$ NK cells. The HER-2 transfectants were clones chosen for their high (HER-2:L), medium (HER-2:B), or low (HER-2:G) expression of HER-2. **C, D, E** Surface MHC class I, HER-2, LFA-1a (CD11a), LFA-1b (CD18), LFA-3 (CD58), and ICAM-I (CD54) expression by C1R:A2 and HER-2 transfectants was analyzed by flow cytometry

targets. Furthermore, the sensitivity of the clones to lysis by NK cells varied inversely with the density of HER-2 expression; i.e. the sensitivity of the transfectants to lysis ranked G > B > L, while HER-2 expression ranked L > B > G (Fig. 5).

The HER-2:G targets were most sensitive to NK lysis even though they expressed a higher density of HLA-A2 than did HER-2:B and HER-2:L. Additional phenotypic analyses of these clones revealed that they all expressed only very low levels of CD18 (LFA-1β) and CD11a (LFA-1α), while CD54 (ICAM-1) and CD58 (LFA-3) were expressed at similar levels among the cloned transfectants (Fig. 5C–E). Therefore, there was no correlation between adhesion molecule expression and the sensitivity of HER-2 transfectants to lysis by NK cells. Our data suggest that quantitative and qualititative changes in the composition of the naturally processed HER-2 peptides presented by MHC, rather than alterations in the expression of MHC class I or adhesion molecules, are responsible for the protective effects of HER-2.

Discussion

In this report we present novel evidence that HLA-A2-binding HER-2 peptides, known to form CTL epitopes, can protect targets from lysis by NK cells. This protection was found to be dependent upon (a) peptide concentration, requiring pulsing with peptides at 50–100 μg/ml; (b) peptide sequence, since single amino-acid substitutions could significantly alter the status of target susceptibility; and (c) side-chain charge, with charged side-chains at position 1 generally inducing more protection from NK lysis than uncharged side-chains. In support of previous studies by others [2, 25, 26, 28, 43], this indicates that NK cells recognizing peptide-MHC complexes display a high degree of target specificity. These findings also suggest that CTL epitopes on tumor cells may block NK lysis, a mechanism that may have implications for tumor survival in the absence of CTL. An increase in the relative ability of a peptide to inhibit lysis was, in most cases, not associated with increased expression of HLA-A2 on T2 target cells, or with conformational changes of HLA-A2 detected by BB7.2 and MA2.1, suggesting that these serological epitopes are not solely responsible for inhibition of NK function.

HLA-A2 conformational changes were often seen on targets that were most sensitive to lysis in this study. For example, increased staining with the BB7.2 mAb was associated with enhanced lysis in the case of the T1 peptide and decreased protection from lysis for E75. One possible explanation for the enhanced sensitivity to lysis of targets bearing HLA-A2 conformational changes could be that, although HLA-A2 expression inhibits lysis, it can only do so if the conformation is not altered by the peptide. However, the full explanation is probably more complex, because E89 induced a fair amount of HLA-A2 conformational changes (both MA2.1 and BB7.2) yet inhibited lysis as effectively as C85, a peptide that did not induce such changes in HLA-A2. One alternative explanation for the enhanced sensitivity to lysis caused by T1 is the hydroxylated side-chain (tyrosine) at P1, which may have decreased the recognition of HLA-A2–peptide by an inhibitory NK receptor. Further experiments are necessary to elucidate this mechanism.

In agreement with previous studies, the peptide concentrations required to induce a significant NK-protective effect were higher than the concentrations required to sensitize T2 cells to CTL effectors from breast and ovarian cancer patients [12, 14]. This may indicate that these effects are only relevant in vitro. However, recent studies on peptide binding to HLA-A2 molecules indicate that, during 4–6 h of incubation, the number of class I MHC complexes formed with similar amounts of exogenously added peptides is in the range of 10^3–10^4, which is consistent with the level of expression of a number of endogenous peptides [17]. Therefore our results should be relevant to certain pathological conditions, such as viral infections and cancer, where large amounts of viral or tumor peptides are processed and presented by MHC class I. The observation that NK cells were less effective in lysis of C1R:A2 cells expressing high levels of HER-2, than of those expressing lower levels, is suggestive of this possibility. Thus, protection from NK-mediated lysis may be dependent not only on the presence of self-peptides or MHC, but also on the high-density expression of specific peptide-MHC complexes. These findings are compatible with the use of an NK-inhibitory receptor with low affinity for the recognition of peptide-MHC complexes. Furthermore, the same peptides were capable of inhibiting lysis of HLA-$A2^+$ T2 cells by NK effectors from both HLA-$A2^+$ and HLA-$A2^-$ donors, indicating that the receptor(s) responsible for this inhibition are expressed independently of HLA-A2 expression in the donors.

These studies were performed using highly enriched (up to 98% purity) NK cells, to exclude a role for T cells in any of the observed effects. We also observed that HER-2 peptides protected targets from lysis by an established NK cell line. In no experiment, though, was complete protection of T2 cells by HER-2 peptides observed. This is not surprising, because the NK cells used in our studies were not clones. It has been shown that different NK clones can respond differently to the same peptide-pulsed targets [8, 9, 23, 25], most likely because of expression of different combinations of inhibitory and activation receptors. Bulk NK populations were used in most of our experiments to mimic more closely the effector/tumor conditions existing in vivo. In fact, it is important to realize that the 15%–30% of tumor cells that might be protected from NK cells by HER-2 peptides would represent a substantial number of malignant cells likely to escape NK cell attack.

Our results show that a side-chain charge at P1 of two different HER-2 peptides is important for protection from lysis. It is interest that the requirement for a specific side-chain in the protection of a target against NK-mediated lysis suggests that certain NK receptors, or

structures on NK cells involved in target lysis, directly contact MHC-bound peptide. Importantly, these effects were observed for the first time when peptides known to induce CTL-mediated lysis in the HLA-A2 system were used. Recent studies have shown sequence-specific NK-potentiating effects for P8 of nonapeptides, although the effects were not associated solely with charged residues at P8 [26]. Furthermore, Peruzzi and collaborators identified a role for P7 and P8 of HLA-B*2705-associated peptides in modulation of NK recognition [28]. Charged side-chains in residues at P7 and P8 in their system enhanced NK-mediated lysis. These studies indicate that residues in certain positions of the class-I-MHC-bound peptides can up- or down-modulate NK lysis. Nevertheless, the effects may be dependent upon HLA type or other unknown factors, which may help explain why one donor of four tested (Fig. 3) in our study showed a somewhat different-from-average pattern of NK inhibition by the C85 variants (inhibition by F1 and G1 but not K1; data not shown). It is most likely that HER-2 peptides were inhibiting lysis directly through the interaction of HLA-A2–peptide complexes with NK receptors, since A2-specific mAb significantly blocked the inhibition. Although the inhibition was not completely blocked when high levels of peptide were used, likely explanations are that monomorphic HLA-A2 was up-regulated more than the MA2.1 conformational epitope or that the antibody was not saturating the HLA-A2 at high peptide concentrations. This could also possibly be due to peptide stabilization of non-classical MHC, such as the deletion variants described by Abu-hadid et al. [1].

Positive stimulation (activation) of NK cells may occur through several different activation or costimulatory receptors on NK cells, such as NKR-P1 proteins, CD16 and CD28, but it appears that the specificity of NK target recognition is often not provided by activation signals, but rather by the presence or absence of inhibitory signals induced by recognition of peptide-MHC complexes [20]. It has been suggested that peptide-induced protection from NK cells may be due to stabilization and/or conformational effects of peptides on MHC class I molecules. However, the role of the peptide in NK recognition is probably not simply to stabilize MHC class I or to promote changes in MHC conformation. NK cells express an array of different receptors that inhibit target cell lysis upon recognition of MHC class I. Examples are the C-type lectin superfamily of receptors (e.g. CD94, NKG2) and the killer-cell-inhibitory receptors of the immunoglobulin superfamily (e.g. p 70, p 58) [4, 20, 29]. Several investigators have now demonstrated that inhibitory receptors on NK cells not only recognize specific types of MHC but also recognize a specific subset of peptides on HLA-B or C [4, 25, 28–30, 43]. Our results in the HLA-A2 system also show that NK cell recognition is sensitive to mutations in peptides that minimally affect monomorphic MHC class I expression. Furthermore, changes in the expression of conformational MHC epitopes did not appear to cause the inhibition of NK-mediated lysis in this model, although such epitopes may have caused increased sensitivity to lysis, as discussed above. It is tempting to hypothesize that NK receptors use a similar mechanism of recognition to the one recently proposed for the T cell receptor [10]; i.e., the proper conformation of the MHC-peptide complex is required for the receptor to "land" on the target, while the changes in side-chain moieties (charge, polarity, van der Waals forces), are responsible for initiation of signaling. This will explain why expression of the MA2.1 conformational epitope does not correlate with recognition, since the epitope recognized by MA2.1 mAb is directly affected by side-chains of residues in pocket A (and possibly B) of HLA-A2, while the BB7.2 mAb detects altered conformation induced by the peptide in a different position (α2 domain, W108), which does not interact directly with peptide side-chains. More extensive studies are needed to address this point, but this study suggests that a number of mutations in peptides (including CTL epitopes) presented by MHC class I may interfere with MHC recognition by NK cells. These findings may have implications for understanding the mechanism by which cells infected with viruses (e.g. influenza or AIDS), and displaying a high rate of mutation, might escape immune defenses. This mechanism may also apply to tumor cells where overexpression of certain gene products (e.g. tyrosinase, gp100, or Muc-1) could lead to the presentation of a high density of self-epitopes with inhibitory effect on NK cells. An additional possibility to be examined is that presentation of mutated peptides (e.g. from p53 or p21) may protect tumor cells from NK surveillance.

In support of our conclusions, it has been shown previously that HER-2-overexpressing breast and ovarian cell lines were more resistant to NK-mediated lysis than nonexpressing (or HER-2^{low}) targets [21]. As was the case also in our investigations, resistance in the latter studies could not be attributed solely to an increase in MHC class I or to changes in ICAM-1 expression by the HER-2^{+} targets [11, 22]. Taken together, these results suggest that endogenously processed HER-2 peptides expressed in complexes with MHC class I molecules may contribute to the resistance of HER-2-overexpressing tumor cells to NK-mediated lysis. Therefore, further elucidation of how NK cells recognize peptides may help to explain the aggressiveness of some tumors, as well as provide new insight into the nature of NK cell receptors for antigens.

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Folate Binding Protein Peptide 191-199 Presented on Dendritic Cells Can Stimulate CTL from Ovarian and Breast Cancer Patients

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Abstract. *Tumor associated lymphocytes (TAL) isolated from malignant ascites cultured in media containing interleukin-2 show antitumor responses. These antitumor responses are mediated by cytotoxic T lymphocytes (CTL) which recognize antigen in the context of MHC molecules using T cell receptors. CD8+ CTL recognize peptide epitopes processed from cellular proteins in the context of MHC class I molecules. These peptides have a restricted length of 8-11 amino acids. The folate binding protein (FBP) is overexpressed in over 90% of ovarian and 20-50% in breast cancers. We recently found that FBP is the source of antigenic peptides recognize by a number of these CTL-TAL. This indicated that FBP peptides are antigenic in vivo for ovarian and breast CTL-TAL. To define FBP immunogenicity, a peptide defining the epitope E39 (FBP, 191-199) was presented by PMBC derived dendritic cells (DC) from healthy donors isolated by the CD14 method to ovarian and breast CTL-TAL. Stimulation of ovarian and breast CTL-TAL by E39 pulsed DC (DC-E39), in the presence of IL-2, rapidly enhanced or induced E39 specific CTL activity. This E39-responder population consisted of cells expressing TCR Vβ9, Vβ13, and Vβ17 families, based on the increase in the percentages of these families in DC-E39 versus DC-NP stimulated TAL. Characterization of immunogenic tumor antigens and of cytokine requirements for induction of functional antitumor effectors may be important for future cancer vaccine developments.*

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Key Words: Folate binding protein, FBP, T-cell receptor, TCR, cytotoxic T-lymphocytes, CTL, tumor associated lymphocytes, TAL, ovarian cancer, breast cancer.

The identification of tumor antigen (Ag) recognized by CTL in melanoma as well as in other cancers such as ovarian cancer raised interest in developing novel molecular therapies for cancer based on tumor Ag stimulation of CTL [1,2]. Since the tumor Ag recognized by CTL consist of short amino acid sequences (8-11 residue long), which define epitopes presented by MHC-I molecules, the central hypothesis of all these studies is that these specific sequences can induce anti-tumor CTL immunity. Definition of the immunogenicity of these epitopes is based on their ability to stimulate CTL in vitro and in vivo to expand and express specific CTL function [3]. Although T cell stimulation and vaccination with short defined sequences it is expected to overcome concerns of specificity of recognition and to focus the responses to well defined epitope, tumor specific CTL stimulation/induction by short peptides has encountered difficulties [4-7]. This was expected given the reported complexities in inducing CTL capable of recognizing endogenously presented Ag, at stimulation with exogenously added nonamer peptides [8,9]. In general exogenous peptides pulsed on various APC poorly stimulated CD8+ cells from PBMC, and lead to CTL that recognized at higher extent the exogenous but not the endogenous presented Ag. This phenomenon was more frequently described in extensive studies in the melanoma system, where patients vaccinated with wild type melanoma gp100 peptide 209-217, or with a mutated analog g9-209(2M) induced high numbers of peptide 209-217 specific CTL but weak clinical responses [10,11]. Further analysis demonstrated that such CTL recognized the wild type or the mutated analog but poorly the gp100 expressing melanoma cells.

Similar results were obtained in in vitro and in vivo studies in other systems, such as ovarian carcinoma using

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HER-2 peptide E75 immunogen and incomplete Freund£s adjuvant [12]. This was not unexpected since it has been previously shown by studies with tumor Ag that numerous repeated peptide stimulations are necessary for expanding Ag or tumor specific CTL from PBMC [13-14]. Furthermore DC peptide stimulation can more easily expand activated [15] than primary effectors [16].

Ongoing studies are focussed on approaches to overcome the poor immunogenicity of the tumor Ag when delivered in peptide form. One of these approaches uses DC as APC. This aims to enhance the peptide immunogenicity by increasing both the Ag levels and the levels of co-stimulatory molecules. This is because DC have the capacity to uptake higher amounts of peptides than other APC. While the DC approach appears to require less cycles of stimulation for CTL induction than the PBMC as APC. its use for therapeutic purpose depends on the availability of DC precursors. This is an important issue for cancer patients particularly for the ones with advanced disease with low blood counts and functionally impaired DC [17,18].

The fact that PBMC derived DC cultured in GM-CSF plus IL-4 show poor proliferation and limited life span [19], raised the possibility of using as APC, DC from healthy donors for stimulation of CTL. Tumor infiltrating lymphocytes (TIL) and/or TAL show a higher frequency of Ag-specific CTL than PBMC and consist of activated memory effectors [20]. This raised the possibility of stimulating TIL/TAL with peptide pulsed DC to expand Ag-specific clonal populations, on the rationale that lower Ag concentrations and less co-stimulatory interactions are needed for activation of memory than of naove T cells.

To investigate this possibility, we stimulated ovarian and breast TAL from six distinct patients with peptide pulsed allogeneic DC. The peptide used for stimulation correponded to a immunodominant CTL epitope mapping of the amino acids, 191-199 of the FBP, a newly identified tumor Ag [21]. FBP is overexpressed in the majority of ovarian (over 90%) and 50% of breast cancers [22-25]. The DC were generated from healthy donors that were HLA.A2 matched with the patients. The results showed that E39 specific CTL could be easily generated from TAL from patients with advanced disease. These peptide E39 stimulated TAL recognized autologous tumors. DC-E39 stumulation lead to expansion of certain clones from the TAL population as illustrated by the preferential increase in population of CD8+ cells expressing certain TCR Vß families.

This increase was not due to allo-stimulation since TAL from the same patient stimulated with the same DC in the same experiment but in the absence of E39 failed to induce E39-specific CTL and specific increase of the same TCR Vß familes. These findings may be significant for therpeutic approaches for ovarian and breast cancer patients with advanced disease. This may be useful for *in vitro* immunization and expansion of CTL of desired specificity followed by adoptive immunotherapy.

Materials and Methods

Cytokines. The following cytokines were used in this study: GM-CSF (Immunex corp., Seattle), specific activity 12.5 x 10^7 CFU/250mg, IL-4 (Biosource International), specific activity 2 x 10^6 U/mg, IL-2 (Cetus, Emeryville, CA), specific activity 4 x 106 BRMP U/mg, IL-15 (Genzyme, Cambridge, MA), specific activity 2 x 10^6 U/mg.

Synthetic peptides. Peptides were synthesized in the Synthetic Antigen Laboratory of U.T. M. D. Anderson Cancer Center using solid phase techniques on an Applied Biosystems 430 peptide synthesizer (Applied Biosystem, Foster City, CA). Identity and purity of final material were established by amino acid analysis and analytical reverse phase HPLC (Rainin). All peptides utilized in this study were between 92-95% pure. Two FBP peptides were selected for synthesis based on the presence of leucine, isoleucine or valine in the dominant anchors position. As their previously reported recognition by TAL the peptides position and sequence are as follows: E39 (FBP, 191-199) EIWTHSTKV; E41 (FBP, 245-253) LLSLALMLL. Both peptides are low to moderate binders to HLA-A2 [21].

Cells. For induction of dendritic cells in the presence of cytokines GM-CSF and IL-4, HLA.2+ PBMC were obtained from healthy donors from the Blood Bank of M.D. Anderson Cancer Center. For generation of DC by the CD14 method, PBMC were distributed in 24 well plates at 4 x 10^6 cells/well in RPMI 1640 medium. After 2 h of incubation, the non-adherent cells were removed. Complete RPMI medium containing 1000 U/mL GM-CSF and 500 IU/mL IL-4 was added to each well and the adherent cells were cultured for 5-7 days, while they developed the DC characteristic morphology.

Tumor Associated Lymphocyte Cultures. TAL were isolated from fresh collections of malignant ascites and pleural effusions from 4 ovarian and 2 breast cancer patients from the departments of Gynecologic Oncology and Breast Medical Oncology at U. T. M. D. Anderson Cancer Center under the approval of the Institutional Review Board. Specimens were processed as we described [26]. The suspensions of the lymphocytes and tumor cells were separated by centrifugation over discontinuous 75% and 100% Ficoll-Histopaque (Sigma, St. Louis, MO) gradients. Freshly isolated TAL were cultured in RPMI 1640 containing 100 (g/ml L-glutamine (Gibco, Grand Island, NY) supplemented with 10% FCS (Sigma), 40 (g/mL gentamicin (complete RPMI medium), and 50 to 100 IU/mL IL-2 (Cetus, Emeryville, CA). TAL were cultured at 0.5 to 1.0 x 10^6 cells/mL, placed in a humidified incubator at 37°C in 5% CO_2 and maintained at this concentration with the addition of media and IL-2 every 2 to 3 days, depending on the growth kinetics.

T cell stimulation by peptide pulsed DC. DC were washed three times with serum free medium, plated at 1.2 x 10^5 cell/well in 24-well culture plates and pulsed with FBP peptide. E39, at 100 µg /ml in serum free medium for 4 hours before addition of responders as described [27]. These DC were designated as DC-E39. Paralled control DC cultures were established and maintained in the exact same manner except for the omission of FBP peptide (designated DC-NP). The responder TAL in complete RPMI medium were added to DC at 3 x 10^6 cells/well (stimulator : responder ratio of 1:25). 16 hours later IL-2 was added to each well at a final concentration of 30IU/ml and the cultures were left undisturbed for the following 5 days when CTL activity was determined.

Tumor targets. The FBP + ovarian SKOV3 line was transfected with the HLA-A2 expression vector RSV.5-neo with resulting high levels of

HLA-A2 expression (SKOV3. A2) as previously described [26] and maintained in complete RPMI medium and 250 μg/mL G418 (Sigma). Fresh tumors were collected from the malignant ascites after Ficoll separation and frozen in aliquots in liquid nitrogen until used.

Phenotype Analysis. The HLA-A2 status of the TAL lines and tumor cell lines was determined by indirect staining with anti HLA-A2 mAb BB7.2 (ATCC) followed by incubation with goat anti-mouse mAb conjugated with FITC (Becton Dickinson, Mountain View, CA) and analyzed on a Coulter Epics C Cytometer (Coulter Electronic, Hileah, FL). FBP expression was analyzed using the Mov18 mAb generously donated by Centocor (Malvern, PA).

Flow cytometry for TCR Vβ expression. TAL were stained with fluorescein and phycoerythrin-conjugated mAb specific for the TCR Vß families. The following mAbs were purchased from Pharmingen (San Diego, CA) and Endogen (Wolburn, MA). Vß3.1, Vß5a, Vß6.7, Vß8a, Vß9, Vß12, Vß13, Vß17, Vß23. The normal mouse IgG1 and IgG2a of Ig isotype were used as isotype controls. Two-color flow cytometry CD8:TCR Vß was performed using a FACScan (Becton-Dickinson) as described [28]. Since there are more than 20 Vß families, the average percent expression of each TCR Vß family should be in the range of 4-5%. We considered a significant increase in the percent Vß for each family when the difference between percent Vß of DC-E39 stimulated and DC-NP stimulated was higher than 5-10%.

Cytotoxicity assays. Recognition of peptides used as immunogens was performed by standard chromium release CTL assay as described [26,29]. T2 or tumor targets were labeled with 200 μCi of sodium chromate (Amersham, Arlington Heights, IL) for 1.5 hrs at 37°C, washed twice and plated at 3000 cells/well in 100 μl in 96 well V-bottom plates (Costar, Cambridge, MA). Effectors were added at designated effector : target (E:T) ratios in 100 μl/well. After 5 h of incubation, 100 μl of culture supernatant was collected, and ^{51}Cr release was measured on a gamma counter (Gamma 5500B, Beckman, Fullerton, CA). All determinations were done in quadruplicate. The results are expressed as percent specific lysis as determined by the equation : (experimental mean cpm - spontaneous mean cpm) / (total mean cpm - spontaneous mean cpm) x 100. For peptide-pulsed cytotoxicity assays, the T2 cells were labeled as above, washed, and then incubated either with PBS (T2-NP) or with peptides (DC-E39) for 1.5 hr at 37°C before standard CTL assays were performed.

Monoclonal antibody blocking assay. Effectors were incubated with anti-V beta mAbs Vß3.1, Vß5a, Vß6.7, Vß8a, Vß9, Vß12, Vß13, Vß17, Vß23. (50 μL of 1:50 dilution of culture supernatant/well) for 30 minutes at 37°C before being added to the standard CTL assays then the assays were performed as described above.

Cold target inhibition assays. Unlabeled T2 were incubated with E39 for 1.5 hr, then added to standard CTL assays with chromium-labeled tumor targets and effectors. The cold : hot target ratios were 10:1 and 20:1. The T2-NP were used as a control.

Results

Patients characteristics. Four ovarian and two breast cancer patients were selected for this study. The ovarian TAL (OTAL) and breast TAL (BTAL) were isolated from the malignant ascites and pleural effusion specimens. They were all found to be HLA.A2+ and the CD8+cells in these ascites ranged between 20-40%. The patients ages ranged between 45 to 63 years and the stages of diseases were all far advanced.

Table I. *The clinical characteristics of the patients.*

	Ovarian cancer			Breast cancer		
	Pt.1	Pt.2	Pt.3	Pt.4	Pt.1	Pt.2
Stage :	IIIc	IIIc	IIIc	IIIc	IIIb	IIIa
Age :	52	50	45	47	63	56
Histology :	AC	PSA	AC	PSA	IDC	IDC
Grade :	III	III	III	III	III	III
1st Tx :	TRS	TRS	TRS+BSO	TAH+BSO	MRM	RM
2ndTx :	PC	PC	PC	PAC	Taxol	CMF+CAP
Other Fx :		ER+,PR+	BrestCa		ER+,PR+	
Prognosis :	Poor	Poor	Fair	Poor	Poor	Poor

AC, adenocarcinoma; PSA, papillary serous adenocarcinoma; IDC, infiltrating ductal carcinoma; TRS, tumor reductive surgery; TAH + BSO, total abdominal hysterectomy + bilateral salpingooophorectomy; MRM,modified radical mastectomy; RM, radical mastectomy; PC, carboplatin + taxol; PAC, carboplatin + adriamycin + cytoxan; CMF, cytoxan + methotraxate+ 5-FU; CAP, Cytoxan + adriamycin + cisplatin

According to cell types all six patients had highly differentiated cell grade and for the histology, two ovarian cancer were adenocarconoma, two were papillary serous cystadenocarcinomas and two breast cancers were infiltrating ductal carcinomas. They all received primary cytoreductive surgery follwed by adjuvant chemotherapies. The survival period for the study subjects ranged from 22-69 months for the ovarian cancer and 12-38 months for the breast cancers from the diagnosis of disease to study initiated. A brief description of patients chracteristics is summarized in Table I.

Freshly cultured ovarian TAL recognize FBP peptide E39 after stimulation with DC-E39. Fresh isolated TAL cultured in media containing IL-2 express either low levels of Ag specific cytotoxicity or high non-specific lytic activity during the first 7-10 days of culture. Although the non-specific cytolytic activity decrease over time, it is important to identify approaches to enhance specific CTL activity early and rapidly. This study was focussed on TAL samples which showed either low levels of specific recognition of E39 or E39 recognition was non-specific compared with T2-NP. Representative results for all samples are shown in Figure 1A. To examine the specific recognition of FBP peptides, we cultured isolated OTAL1 in IL-2 without specific stimulation for 1 week. Then the OTAL1 was tested at two different E:T ratios in 5-h chromium release assays for recognition of peptides presented by E39 and E41 T2 cells. As demonstrated in Figure 1A the results showed preferential recognition of E39

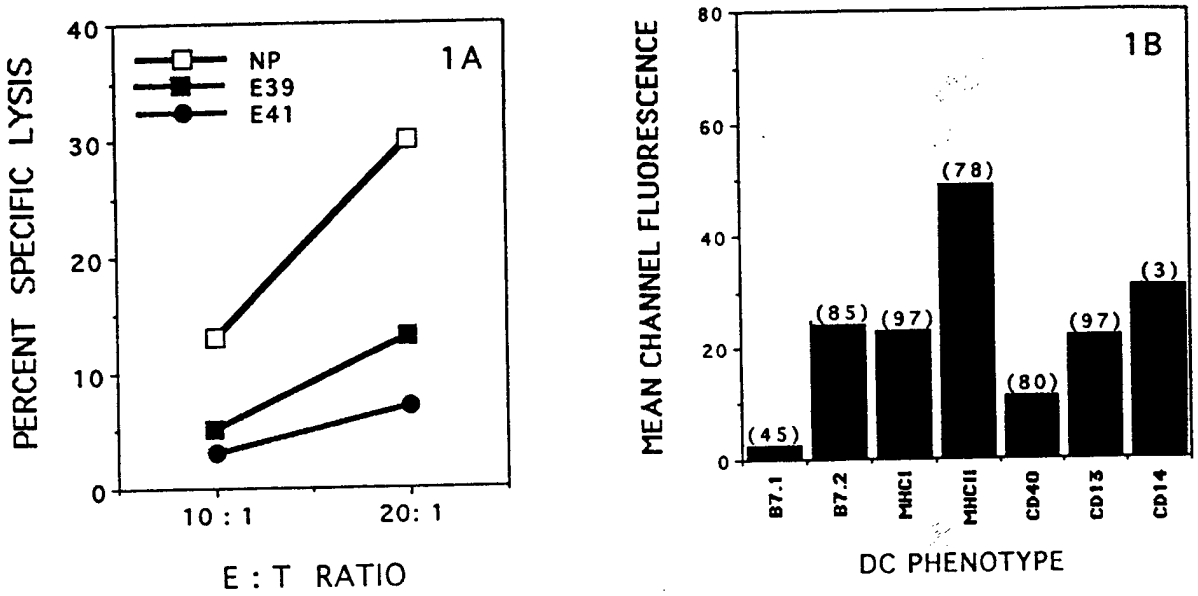


Figure (1A). *Freshly cultured ovarian TAL1 recognize FBP peptide E39. Fresh isolated TAL cultured in IL-2 expressed high levels of non-specific Ag cytotoxicity. The 5-h Cr release assays for the recognition of peptides E39 and E41 cells showed preferential recognition of E39 over E41. Experimental details are presented in the material and methods section. (1B), Cell surface phenotype of DC generated after GM-CSF + IL-4 cultured. PBMC derived DC express high levels of MHC-I, MHC-II and CD86 (B7.2) but low levels of B7.1 (CD80) and CD40. The CD14+ cells were less than 3% of DC, while the CD13+ marker was expressed on more than 97%. Parentheses ( ) indicate the percent positive cells.*

over E41. However OTAL1 showed significantly higher recognition of DC-NP comparing with DC-E39, suggesting a high percentage of non-specific or cross reactive lytic effectors.

To determine whether the E39 specificity can be induced or enhanced we used as APC HLA.A2+ matched dendritic cells (DCs) from healthy donors. The phenotype of DC generated after GM-CSF + IL-4 is shown in Figure 1B. They expressed high levels of MHC-I and CD86 (B7.2) but low levels of B7.1 and CD40. The CD14+ cells were less than 3% of DC, while they expanded the CD13+ marker was expressed on more than 97% cells. This phenotype is characteristic of immature DC. We pulsed DCs with the peptide E39 and then used DC-E39 to stimulate the OTAL. Since the responders and stimulators were from different individuals which shared only HLA. A2 a certain level of allo-specific and/or cross-reactive specificity was expected. Therefore in all experiments the OTAL and BTAL were stimulated in paralled with DC-NP. The parallel stimulations with DC-NP and DC-E39 were done to established the contribution of allospecific responses to the overall increase in lytic activity. Furthermore, if high affinity E39-specific CTL were present and they would have been deleted by DC-E39 stimulation, they would have been detected in the DC-NP stimulated cultures.

It was interesting to find that, in most TAL the E39 specificity was induced at the first stimulation. When increased E39 specific recognition was not induced instances at the first DC-E39 stimulation it was induced at restimulation. For example at the first stimulation with DC-E39, the specific lysis of DC-NP vs DC-E39 by OTAL2 were 13.2% vs 15.1% respectively, (Figure 2A). When we restimulated the same OTAL2 with DC-E39 again, for 1 more week, we observed a significant increase in DC-E39 recognition compared to DC-NP : 25.7% vs 15.3%, ($p < 0.0002$) (Figure 2B).

Increased CTL-mediated Cytotoxicity of FBP peptide E39 stimulated TAL. E39 (FBP, 191-199) appears to be the immunodominant FBP epitope. CTL assays were performed to determine whether the DC-E39 stimulation increased the levels of recognition of the stimulating antigen. To address this question, we used E39 pulsed T2 as targets. The CTL assays were performed with four ovarian and two breast TAL as effectors at an E:T ratio of 20:1. The results demonstrate that after DC-E39 stimulation all four ovarian TAL and one of two breast TAL showed specific recognition of E39 compared to DC-NP, (Figure 3A). OTAL1 and OTAL3 showed highest specific lysis compared to control. 73.6% and 29.6% respectively, followed by OTAL2 and OTAL4 as 25.7% and 41.8% respectively. These differences were all statistically significant compared to controls. ($p < 0.05$). These results, also demonstrate that stimulation with DC-E39 resulted in the highest levels of cytotoxicity against E39 in

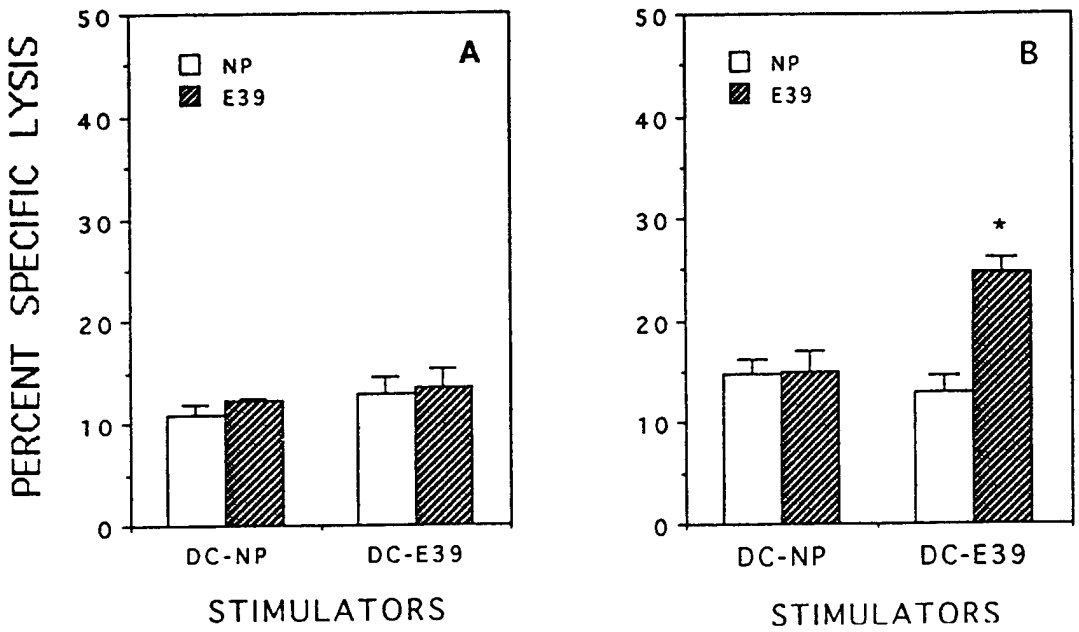


Figure 2(A,B). *Induction of E39 specificity in the ovarian TAL2 require restimulation with DC-E39. Figure (2A), in most TAL the E39 specificity was induced at the first stimulation. OTAL2 required restimulation as shown here. At first stimulation, the specific lysis of OTAL2 stimulated with DC-NP vs DC-E39 were 13.2% vs 15.1% respectively. Figure (2B), when we restimulated OTAL2 with DC-E39 again after one more week, we observed a significant increase in E39 recognition by DC-E39 stimulated OTAL2 compared to DC-NP, 25.7% vs 15.3%. ($p < 0.0002$). (Figure 2B).*

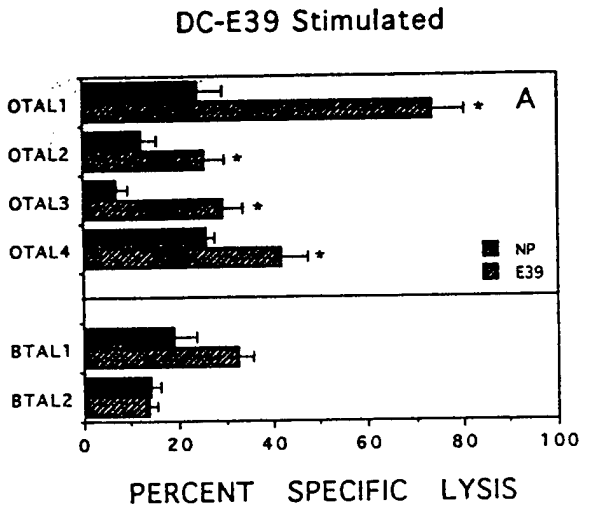
OTAL but not in BTAL. Control stimulation with DC-NP, representive of allo-stimulation alone did not induce or enhance the specific recognition of E39 (Figure 3B). This is equally true for both TAL where the E39 specificity was present (OTAL1, BTAL2) or abscent. These results together with the results in Figure 3A demonstrate the induction of E39 specific CTL-TAL recognition by Ag stimulation. These results are summarized in Table II. Our results also demonstrate that E39 specific CTL were consistently induced in ovarian TAL but not in the breast TAL.

T Cell receptor Vβ expression of TAL stimulated with FBP peptide E39 pulsed DC. We wanted to investgate whether DC-E39 stimulation induced specific changes in the TCR Vß expression. This was achieved by comparing specific TCR Vß expression in DC-E39 versus DC-NP stimulated TAL. To determine the TCR Vß expression in TAL isolated from the ovarian and breast cancer patients, we used monoclonal antibodies (mAb) to stain for 9 different TCR Vß families. Vß3.1, Vß5a, Vß6.7, Vß8a, Vß9, Vß12, Vß13 and Vß23. After 5 days stimulation with DC-E39 and DC-NP two color FACScan analysis was performed for CD8 and Vß expression. In this part of the study, we wanted to investigate the expression of various TCR Vß families in TAL populations from distinct patients stimulated with DC-E39. Complete results for TCR Vß expression by OVTAL1 are shown in Figure 4. Among the 9 different TCR Vß families tested, we found that the expression of levels of only two Vß families

Table II. *The E39 reactivity CTL Ftesponse according to TCR Vβ expression on TAL from ovarian and breast cancer patients.*

	Specific E39 reactivity		
	Before	After	Increased
Ovarian TAL			
Patient 1	+	+	↑
Patient 2	-	+	↑
Patient 3	-	+	↑
Patient 4	-	+	↑
Breast TAL			
Patient 1	-	+	↑
Patient 2	+	-	-

was significantly higher (by more than 5% increase) than the one induced by DC-NP. These families were Vß9 and Vß13. The expression of other V(families (Vß3.1, V(5a, V(8a, V(17 and V(23) showed some increase (by less than 5% V(expression) than the corresponding Vß expression in the DC-NP stimulated cultures. The expression levels of V(6.7 and



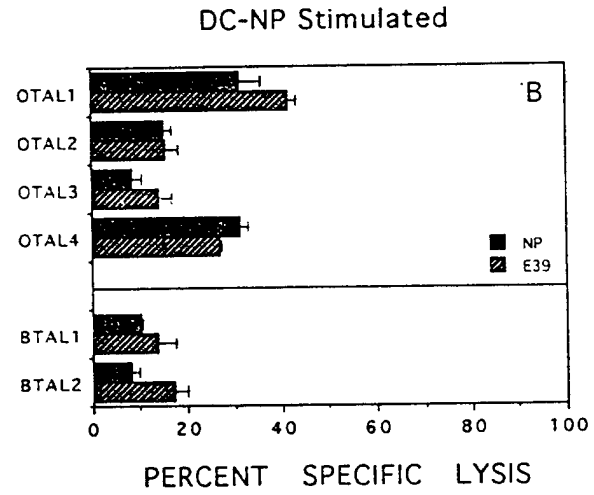


Figure 3. *Increased CTL-mediated recognition of peptide E39 by DC-E39 stimulated TAL. The CTL assays were performed with four ovarian and two breast TAL as effectors at an E:T ratio of 20:1, (Figure 3A). After DC-E39 stimulation all 4 OTAL and 1 of 2 BTAL showed specific recognition of E39 compared to DC-NP. The % specific lysis are OTAL1 73.6%, OTAL2 25.7%, OTAL3 29.6%, OTAL4 41.8% and BTAL1 31.9%. These difference are statistically significant. ($p < 0.05$) Figure 3B. Control stimulation of ovarian and breast TAL with DC-NP did not enhance the specific recognition of E39.*

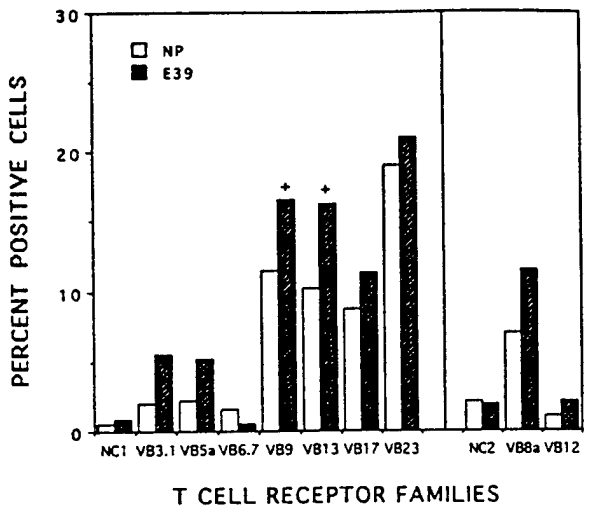


Figure 4. *TCR Vβ expression of OTAL1 stimulated with DC-E39. Among the 9 different TCR V(families tested, we found that the expression of levels of only two Vβ families, Vβ9 and Vβ13 was significantly higher (by more than 5% increase) than the one induced by DC-NP. The expression of other Vβ families, Vβ3.1, Vβ5a, Vβ8a, Vβ17 and Vβ23 showed some increase (by less than 5%) than the corresponding Vβ expression in the DC-NP stimulated culture.*

Vß12 did not changed. Comparative results for six TCR Vß families are showed in Figure 5A.B. The results show significant (> 5%) increase in TCR Vß9, Vß13, and Vß17 in OVTAL stimulated with DC-E39 (Figure 5B) compared with the same TAL cultured and stimulated by DC-NP (Figure 5A). Since each of these families was expressed at levels which ranged between 4-8%, the increase observed was suggestive of the fact that the levels of particular Vß families increased by 80-100%. Therefore DC-E39 significantly enhanced the TCR Vß expression of certain families suggesting that the increase is due to antigen stimulation.

There were also differences in the expression pattern of TCR Vß families in BTAL compared to OTAL. The Vß17 and Vß 23 families were elevated in the BTAL1 after DC-E39 stimulation compared to control, DC-NP stimulated BTAL1, (Figure 5C). Thus the ovarian and breast TAL stimulated with DC-E39, shared the specific increase in Vß17, but the other dominant familes were different: Vß9 and Vß13 in OTAL versus Vß23 in BTAL. These results suggests that E39 stimulated CTL recognition of the epitope formed by E39-HLA. A2 involves TCR elements which are shared by only certain TCR Vß families but not by others. Results from of each individual sample are summarized in Table III.

The Specificity of TAL recognition of the FBP-derived peptide E39. To confirm the involvement of the cells expressing the dominant TCR Vß families, in specific recognition of the HLA-A2/E39 peptide complex on ovarian cancer cell line SKOV3.A2 by CTL, inhibition assays were performed. The anti-TCR Vß antibodies were added at the beginning of incubation of a standard cytotoxicity assay. We choose the OTAL2 as effector in the inhibition assay because of the significant increase in Vß17 (shared with BTAL) and Vß9 (shared with OTAL1). We hypothesized that, if Vß9 and Vß17 TCR and CTL are involved in tumor lysis, adding anti-TCR Vß9 and Vß17 antibodies could competitively inhibit the tumor killing. The results obtained demonstrated successful inhibition of tumor lysis of specific Vß9 and Vß17

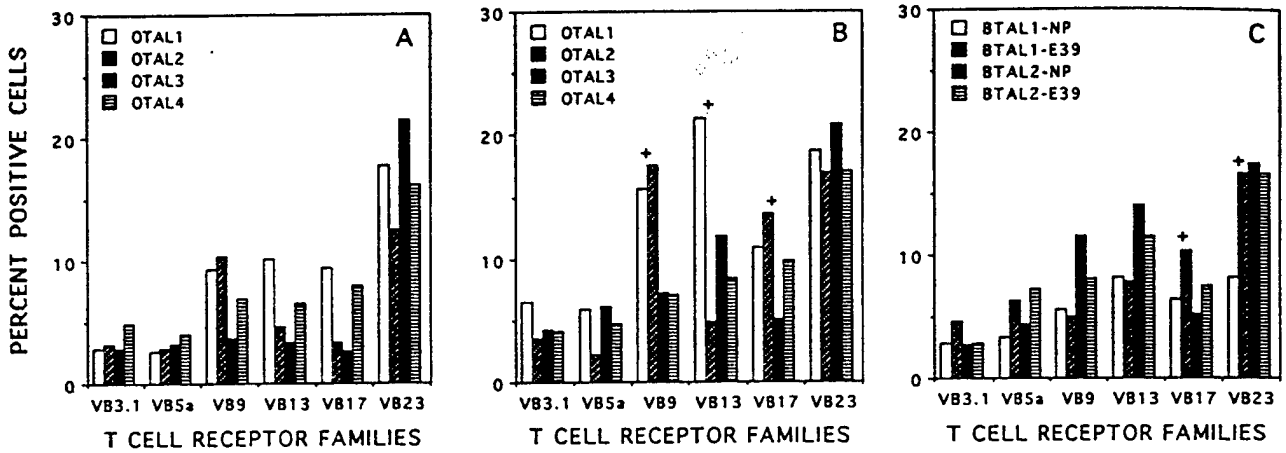


Figure 5. *Comparative expression of six TCR Vβ families by four OTAL (A,B) and two BTAL (C). Significant (> 5%) increase in TCR Vβ9, Vβ13 and Vβ17 in OTAL stimulated with DC-E39 (Figure 5B) compared with the same TAL cultured in the same conditions and stimulated with DC-NP (Figure 5A). There were also differences in the expression pattern of TCR Vβ families in BTAL compared to OTAL. The Vβ17 and V(23 families were elevated in the BTAL1 after DC-E39 stimulation compared to DC-NP stimulation (Figure 5C).*

mAb. Inhibition of OTAL2 lysis was dependent of the concentration of the anti-TCR Vß antibodies added. The anti Vß9 and Vß17 effectively inhibited 40-50% of the tumor lysis. ($p < 0.0002$) (Figure 6A). In contrast, TCR Vß5 and Vß13 antibodies did not inhibit CTL lysis. These findings suggest that the CTL specific for this epitope expressing TCR Vß9 and Vß17 antibodies contribute significantly to the recognition of this ovarian cancer cell line.

To confirm that the peptide stimulated TAL recognized an endogenous presented epitope, we performed cold-target inhibition assays using autologous tumor as target, and T2-E39 as inhibitors. The results show that DC-NP, stimulated OTAL2 did lysed only marginal autologous tumor. In contrast DC-E39 stimulated OTAL2 exhibited significantly higher (2-3 fold) levels of lysis ($p < 0.05$). This lysis was inhibited to the levels induced in the DC-NP by T2-E39. These results indicate that DC-E39 stimulated ovarian TAL recognize similar epitope with E39 on their autologous tumor (Figure 6B).

Discussion

In the present study we investigated whether the FBP peptide E39 a novel tumor antigen, can be used as an immunogen for activation of ovarian and breast CTL-TAL. The important function of TAL is to lyse tumor cells. If E39 specific CTL are present in the ovarian TAL, stimulated CTL-TAL can specifically recognize E39 as lyse experimental tumors. However, stimulation and/or restimulation with peptide pulsed DC may also induce apoptosis or silencing of CTL if appropriate cytokines are absent. For this reason each TAL was stimulated in parallel with DC pulsed with or without E39 to determine whether the E39 specificity decrease or increase. As shown in Table 2, we found that in five out of six patients E39 stimulation resulted in increased E39 specific CTL reactivity. It was interesting to note that, in all four cases of ovarian TAL there was an increase in E39 specific TAL. These responses were obtained in 3 out of 4 ovarian TAL and 1 out of 2 breast TAL by a first peptide stimulation. Only OTAL2 required a second stimulation with DC pulsed with E39 to elicit E39 specific cytotoxicity. The levels of increase in E39 specificity were higher in OTAL1, OTAL2, and OTAL3 and lower in OTAL4 and BTAL1. For the BTAL2 the levels of E39 recognition in fact decreased, suggesting that both activation and functional silencing are possible.

To induce this E39-specific CTL activity we used as APC, DC from four healthy donors. These donors were HLA.A2 matched with the responders and randomly selected so preferential bias in the favor of inducing one phenotype or another could be ruled out. Of the interest, although high levels of allo-specific responses were expected, we found that E39 specificity was higher then the allo-responses.

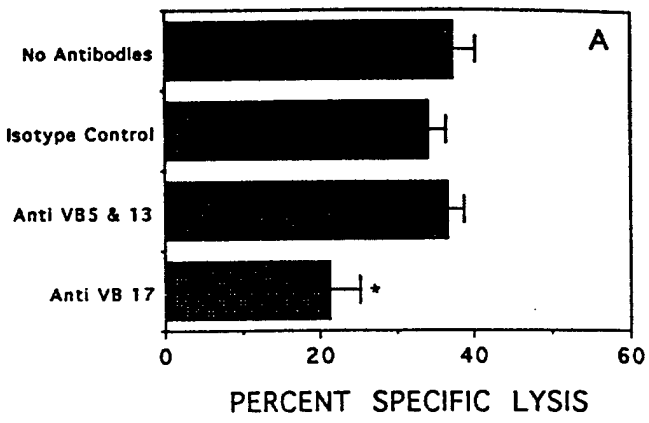
Since the responders and stimulators were only HLA.A2 matched it was interest to determine if the increase the specific lysis correlated with the increase in expression of certain Vß families. Increase representation of Vß families would indicate preferential expansion of a clone or group of clones expressing the same TCR family. Furthermore this increase should be paralleled by a decrease in other families since the sum of Vß families should not exceed 100%. For this reason we compared the % Vß expression in E39 and NP stimulated TAL. The results show that, of nine TCR Vß families tested, only three: Vß9, Vß13, and Vß17 preferentially increased after stimulation with DC-E39.

TCR components that contribute to ovarian tumor recognition by CTL have been studied extensively in past

several years. Most studies were focused on the phenotype analysis of isolated and cultured ovarian and breast TAL with mixtures of $CD8^+$ and $CD4^+$ cells [10, 30, 31]. A previous study by Fisk et al, analyzed the TCR Vß phenotype repertoire of $CD3^+$ $CD8^+$ $CD4^-$ CTL reacting with ovarian tumors [28]. They found significant correlation between percentage of TCR Vß 3 and Vß17 family expression and autologous tumor lysis [28]. Peoples and collaborators also demonstrated that the presence of tumor specific CTL in the TAL of ovarian cancer patients. These CTL recognized shared Ags in an HLA-A2 restricted manner. The antitumor activity was mediated by tumor specific CTL of the V(2, V(3 and Vß 6 families. Vß 3 and Vß 6 recognized TAA that are derived from the HER2/neu gene and presented in the context of HLA-A2 [32]. The TAL tested in previous studies were not stimulated with Ag. In this study, we reach similar conclusions. After Ag stimulation overexpression of TCR Vß 9 and Vß 17 correlated with the increase in specific tumor lysis.

Many immunological studies are focused on ovarian cancer aiming to idenfify the Ag recognized by TAL because it provides an unique model for the study of the immune response to epithelial cancer. Ovarian cancer has distinct forms of tumor growth pattern. It grows either as single cells in the malignant ascites or as a bulky solid mass. In either case, the tumor specifically induce a T cell response. Tumor Ag identification in ovarian cancer is significant and for other epithelial tumors. These epithelial tumors share common CTL recognized TAA and this feature lead to development of TAA specific vaccines. Previous studies demonstrated that, the endogenous cellular immune response does exist in a variety of epithelial cancers, and that this response involves the specific recognition of antigenic peptides presented by MHC-I. Currently the established known tumor Ag are MUC-1 and HER2. MUC-1 expression is increased in 10-40 fold in breast cancer compared to normal cells. HER2 has been shown to provide endogenously recognized antigenic peptides but it is overexpressed in about 30% of ovarian and breast cancers. Therefore it is important to find potentially widely applicable CTL recognized Ag for the development of epithelial cancer vaccines.

FBP was identified with a mAb raised against the choriocarcinoma cell line Lu-75c and independently as the Ag recognized by the MOV18 and MOV19 mAb, and the protein that has high affinity to folate [22-25]. Low expression of FBP was observed in some specialized epithelia, such as choroid plexus, lung, thyroid, kidney, and sweat glands. However the highest levels of FBP overexpression were found in ovarian carcinoma. More than 90% of all ovarian carcinomas tested showed elevated levels of this protein. The elevated levels of FBP are as high as 80-90 folds of that of normal tissue [22-25]. Other epithelial tumors, colorectal, breast, lung, and renal cell carcinoma have been also shown to overexpress the LK26/FBP antigen about 20-50% [22-25]. The variety and widely expressed levels of FBP in various epithelial tumors



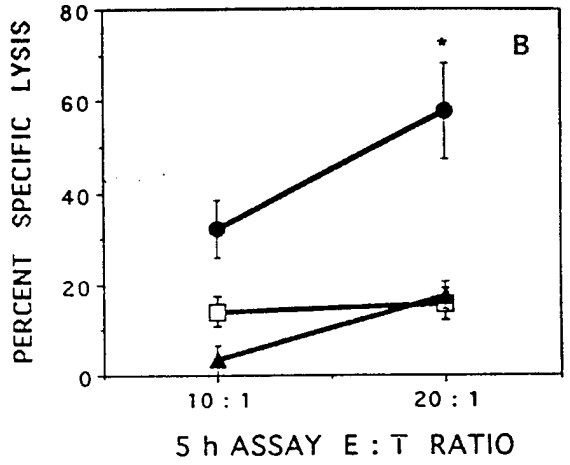


Figure 6A. *DC-E39 stimulated TAL expressing V(17 recognize ovarian tumors. The anti-TCR Vβ antibodies were added at the beginning of incubation of a standard cytotoxicity assay. We choose the OTAL2 as effector in the inhibition assay because of the significant increase in Vβ17 (shared with BTAL). The results obtained demonstrated successful inhibition of tumor lysis by specific anti-V(17 mAb. The anti Vβ17 mAb effectively inhibited 40-50% of the tumor lysis. ($p < 0.0002$). In contrast. TCR Vβ5 and Vβ13 antibodies did not inhibit CTL lysis. Figure (6B), to confirm that the peptide stimulated TAL recognized an endogenous presented epitope, we performed cold-target inhibition assays using autologous tumor as target, and T2-E39 as inhibitors. The results show that DC-NP. stimulated OTAL2 did lysed only marginal autologous tumor.(square in graph). In contrast DC-E39 stimulated OTAL2 exhibited significantly higher (2-3 fold) levels of lysis ($p< 0.05$), (circle in graph). This lysis was inhibited to the levels induced in the DC-NP by T2-E39. (triangle in graph).*

makes an ideal target for the immunotherapy. Inspite of attempts to target FBP antibodies conjugates and antifolates the clinical results were minimal. One reason for the limited efficiency of antibody therapies may be that most antibodies do not recognize FBP in native form, and that FBP has a short extracellular domain which makes the access of

antibody/antifolates difficult. As we demonstrated in this study, if FBP derived peptide, E39, can specifically activate ovarian cancer associated CTL then it can be applied for immunotherapeutic strategies. From previous studies, we know that adoptive immunotherapy can reduce tumor size in some of solid tumors such as melanoma and renal cell carcinoma [36-37], and it can prolong the survival in advanced ovarian carcinoma when combined with conventional chemotherapy[38]. These results were obtained with TIL/TAL. Using specific CTL-TAL directed toward known tumor epitopes such as FBP derived peptide, E39, we can improve the results appreciably. The FBP appears to be the next candidate for the use as target for the cellular immunity.

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